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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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STUDIES UPON THE EFFECT OF LIGHT ON BLOOD AND TISSUE CELLS.

I. The Action of Light on White Blood Cells in Vitro.

By W. R. EARLE.*†

(From the Department of Anatomy, Vanderbilt University School of Medicine, Nashville.)

Plates 9 and 10.

(Received for publication, May 28, 1928.)

INTRODUCTION.

In the course of some work upon photobiological sensitization, Busck (1) found that, upon exposure of blood cells of the dog, suspended in .85 per cent NaCl solution, to light from a carbon arc, all of the red cells were hemolyzed within 15 minutes, whereas the white cells were to all appearances unchanged (Experiment 27, page 491).

Lewis and Lewis (5), from their work on tissue cultures of blood cells from lower vertebrates, dogfish, skate, certain teleost fishes, the frog, the toad, and the snake, make the following statement. "The blood-cultures from these cold blooded animals were kept at room temperature and out of the strong light, as the latter has a decidedly injurious effect upon the cells" (page 102).

In addition to these two, several other references may be cited as of interest. Reed (7), using the carbon-tungsten arc, irradiated the blood of the dog *in vivo*, the light being directed through the wall of a quartz tube inserted in the course of the carotid artery. Directly after the irradiation he found an absolute leucopenia and an absolute lymphopenia, but a relative lymphocytosis.

Miles and Laurens (6), subjected dogs to 1 hour irradiation a day, using a carbon arc which had been so adjusted as to give an intensity of light equal to that of sunlight at Washington, D. C. They found that following irradiation there was a marked rise in the white blood count due to a rise both in the number of neutrophils and of lymphocytes. They also found that in the blood smears

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† Presented to the Graduate School of Vanderbilt University in partial fulfillment of the requirement for a thesis for the degree of Doctor of Philosophy.

there were many "smudges," as well as some lymphoblasts and myeloblasts. They interpret the "smudges" as indicating a rapid destruction of white blood cells.

Clark (2), irradiating the ears of rabbits with light from an iron arc, found that there was an immediate drop in the total white blood count, and that this drop was due to a lymphopenia. Irradiating similar animals through a glass screen she found the total white cell count unchanged after irradiation, but found an absolute lymphopenia.

In an attempt to follow the changes shown by certain leucocytes in tissue cultures[1] of mammalian blood cells, work was greatly hindered by the exceedingly rapid and extreme degeneration of all types of white blood cells in the cultures. In an effort to determine the etiology of this degeneration about 1500 cultures were examined.

Materials and Methods.

The blood from which these cultures were planted was taken from cats, guinea pigs, and rabbits. Most of the work, however, was done on the blood of the rabbit. The general technique used for the preparation of the cultures was as follows. Blood was drawn, either by heart puncture or, after opening the abdomen with the animal under an anesthetic, from the abdominal aorta. It was immediately transferred to oiled or paraffined Pyrex tubes which were stoppered and centrifugated. When the blood clot had formed it was removed from the tube *in toto*, washed in Tyrode solution (pH 7.4), and the buffy coat of the clot was cut up in the same solution, by means of a pair of iridectomy scissors. Fragments obtained in this way were transferred to "non-corrosive" cover-glasses[2] by means of a platinum loop and were then covered by a drop of some one of the following media: Tyrode solution, autogenous serum, autogenous plasma, autogenous heparin plasma, or mixtures of these with chick embryo juice. The cover-glasses were inverted over hollow ground slides of the same glass, and the edges were sealed with "Salvoline," a high melting point petroleum jelly. This whole process was carried out aseptically, and usually required not longer than 1 hour for its completion. These cultures were placed in trays, in groups of five, and set in a dark, water-jacketed incubater which was kept at 37.5°. For examination, a whole tray of slides was removed and set in the microscope hot box which was also kept at 37.5°. Slides were there changed from the tray to the microscope stage as desired.

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[1] The term "culture" is applied to these preparations throughout this whole work, although it is realized that many of the preparations are not true cultures in the sense that they show active growth.

[2] These cover-glasses and slides were secured from Arthur H. Thomas Company.

In the preliminary work, several different microscope light sources were employed. These were all incandescent, tungsten filament, vacuum- or gas-filled, electric globes. They ranged in current consumption from 15 to 200 watts and operated at from 6 to 120 volts. The spectra of two of these sources are shown in Spectra 2 and 3, Fig. 4. These light sources were used either with or without auxiliary glass lenses, and were placed approximately 250 mm. from the concave mirror of the microscope. The light was reflected by this mirror, passed up through the three lenses (1.4 N.A.), aplanatic substage condenser (glass), the hollow ground slide, and the chamber of the slide, thus finally reaching the culture.

EXPERIMENTAL.

An outline of the nature and sequence of the degenerative changes seen in these cultures is presented at this point.

A short time after the microscopic examination of any one of the cultures was begun, it was noted that the neutrophils of that culture rapidly became increasingly ameboid, while the flow of cytoplasm within the cells also increased both in extent and in velocity. At the same time, the cytoplasm became more fluid, as was shown by exaggerated brownian movement of the cell granules. Soon cellular movement ceased and the cells became spherical, although for some time, many of them continued to put out slender cytoplasmic processes. The polymorphic nuclei became swollen and rounded (Fig. 1). As a result of this change in nuclear shape, it was difficult to estimate further changes in the volume of the nuclei, but in many preparations it appeared to undergo reduction after a short time. There was neither shrinkage nor collapse of the nucleus however, until very late in the process of degeneration; rather it remained very turgid. The cells gradually became tremendously swollen; in several instances an increase to three times the normal diameter was observed (Fig. 1). It is of interest to note that this indicates an increase to twenty-seven times the normal volume. During this swelling process the viscosity of the cytoplasm continued to decrease. Further, a number of minute oil droplets, which stained with Sudan III, became visible throughout the cytoplasm. These droplets apparently originated from the cytoplasm.

From this stage on, two terminations of the degenerative process were observed. In the first of these, the cytoplasm gradually coagulated around the nucleus, so that the cell membrane was left as a shell which was slowly autolyzed. In the second, the swelling became so great as to rupture the cell membrane, and a jet of cytoplasmic fluid was thrown out into the surrounding medium. At the moment of rupture the dancing granules and oil droplets rapidly agglutinated, evidently from contact with the surrounding medium. The extruded cytoplasm almost instantly coagulated. Subsequently a process of gradual, and more or less complete autolysis of the dead cell began.

When neutral red or Janus green was added to a culture in any of these terminal

stages, it was found that the cells had completely lost their characteristic vital staining with these dyes. The neutrophilic granules did not show their usual light tan color with neutral red, while, unless the cells were too far degenerate, the nuclei took up the dyes. This reaction is characteristic of dying cells.

The type of degeneration shown by the eosinophil appeared very similar to that shown by the neutrophil. There was the same preliminary acceleration of cellular movement and of intracellular cytoplasmic movement. At the same time, the brownian movement within the cell became exaggerated and the cell began to round up. The brownian movement continued and increased for some time after this, but its amplitude never reached that shown by the granules of the neutrophil. This was due, in part at least, to the large number of granules within the cell, and also to their size.

The eosinophil also became swollen, but even in its final stages its diameter rarely exceeded one and one-half times the diameter of the normal cell. It showed nothing like the amount of swelling shown by the neutrophil, nor was it ever observed to increase in size to the point of rupture of the cell membrane. The nucleus of the eosinophil also increased in size, although this increase, also, was less marked than that noted in the nucleus of the neutrophil. As a result of the swelling, this nucleus likewise had a tendency to become spherical, but even in the extreme stages of its degeneration the two lobes could generally be made out.

The cytoplasm gradually coagulated around the nucleus, and, as a result, one side of the cell was often left almost free of granular material. During this process of coagulation and the still later stages of degeneration, the eosinophilic granules slowly lost their affinity for neutral red, while their outlines became hazy and sometimes completely disappeared. When this occurred the whole group of granules was left as an apparenty free, but coagulated, conglomerate mass. In some cultures these cells remained in this condition for several days. Oil droplets were observed far more rarely in the eosinophil than in the neutrophil, and even in the one or two instances where they were noted, they were very small.

The degeneration of the basophil was essentially like that of the eosinophil, except that the nucleus became enlarged until it was practically spherical, and, since it increased in size far more rapidly than did the volume of the cell, it often forced aside the cytoplasmic granules until they were all crowded within a very small segment of the cell. Only very small fat droplets were observed in the basophil, and these but once or twice.

The monocyte underwent a series of changes almost identical with those shown by the above cells. Within a short time after examination was begun there was a marked increase in the refractive index of the nucleus, this body becoming distinctly visible. As the brownian movement of the cell vacuoles increased, the cell gradually rounded up and its outline became more clearly visible. At the same time, the cell swelled to some degree, and, as indicated by the increased amplitude of brownian movement within the cell, the viscosity of the cytoplasm decreased rapidly. Coagulation later took place. More fatty material was

liberated within the monocyte during its degeneration than within any other type of cell. All of this fatty material, as in the case of that contained within the polymorphonuclear neutrophil, stained deeply in Sudan III.

Because of the difficulty experienced in finding and identifying the large lymphocyte, its degenerative cycle was not worked out. The small and intermediate lymphocytes, however, were more easily followed. These cells were normally almost motionless and no preliminary stimulation, such as was seen in the degenerative cycle of the other cells, was observed. This, however, does not necessarily mean that such does not exist; more probably it means that, in view of the very low motility of the cell and its small area of cytoplasm, our methods for determining stimulation have not been sufficiently sensitive.

The first visible change in the lymphocyte was a marked increase in the size of the cell, often to one and one-fourth to two times its original diameter. The mitochondria, whether examined unstained or stained by Janus green, swelled somewhat, and then gradually disappeared. There was little change in the nuclear size, but the cytoplasm of the cell became typically edematous. In some cases, the cell wall remained for several days as a distinct turgid shell. No fat droplets were observed in these cells.

In connection with the preliminary studies upon which the preceding description is based, several characteristics peculiar to the distribution of degenerate cultures through any one series gave evidence that the degeneration must have been caused by some factor intimately associated with the process of examination. An attempt was next made to examine the different factors which might be responsible for this degeneration. By substitution of a dry objective for the oil immersion objective, the immersion oil was definitely eliminated as the cause of degeneration. Further, by very careful handling of the culture, the factor of mechanical disturbance, consequent to the process of transfer from the incubator to the hot box, and from the tray to the microscope stage, was also ruled out. The temperature of the microscope hot box was next readjusted, being set at 39° (±.1), the blood temperature of the rabbit and the chick. Despite these precautions the number of degenerate cultures was not materially decreased, while the peculiar type of cellular degeneration remained unchanged.

The elimination of these factors, together with the data concerning the distribution of degeneration through any one series of cultures, strongly indicated that the action of light, ultra-violet, visible, or infra-red, either from the surrounding daylight or from the micro-

scope lamp itself, was the most probable agent in causing the degeneration. In order to test out the possibility that light was the causative factor, three series of twenty cultures were prepared. These cultures were inoculated in very dim daylight, and were placed in the dark incubator as quickly as possible. All further handling of these cultures was also done only in very dim light. The individual cultures were taken from the incubator only as needed, placed on the microscope stage with the lamp cut off, and were allowed to remain in the dark for from 5 to 15 minutes in order that the cells might recover from any slight abnormalities due to shaking. Protocols of a group of representative cultures taken from these three series are presented below.

Experiment 1.

Culture 546.—Blood was drawn from a young rabbit under light, ether anesthesia. Cultures were planted in autogenous serum and incubated at 39°. The light source used in the examination of these cultures was the same as for Culture 270. *13.5 hours after inoculation:* Examination and irradiation of the culture were begun. The culture at this time was normal; the cells were moving around slowly. The first observations were made with the substage iris diaphragm closed to its limit, and the light reaching the culture further reduced by means of a Wratten and Wainwright Filter 58 (green). After several minutes examination, the iris diaphragm was opened, but the filter was left in place. The neutrophils almost immediately began moving more rapidly. The green filter was next removed, thus increasing the intensity of light reaching the culture. Brownian movement of the cell granules of the neutrophils began within 10 seconds, and soon became greatly augmented. Within 1 minute cell motion itself had also greatly increased. 1 minute later the filter was again inserted and the examination continued with closed iris diaphragm for 2 minutes. During this time cell movement became slowly reduced, both in velocity and in magnitude. At the end of this time the light was cut off. 1 minute later it was turned on again; during this interval in the dark cell movement had practically ceased, but within a minute or so after the light was turned on it was back to the level at which it was observed at the beginning of the examination. The light was again cut off, this time for 10 minutes, then cut on again. At this time the cells were badly swollen, were rounded up, and cell movement had ceased. The nuclei of the cells were spherical and edematous. There was also a decided increase in the brownian movement of the cell granules. The total time of examination and irradiation was less than 20 minutes.

Cultures 582 and 583.—Blood was taken from the abdominal aorta of a young rabbit under light, ether anesthesia. Cultures were inoculated in autogenous

serum and incubated at 39°. The light source used in the examination of the culture was a 60 watt, 110 volt, vacuum-filled globe. 35 mm. of water was inserted into the optical path. For the spectrum of this light source see Spectrum 2, Fig. 4. *90 minutes after inoculation:* These two cultures were taken from the dark incubator and placed side by side on the microscope stage, in such a manner that while Culture 582 received the full intensity of light coming up through the microscope condenser, Culture 583 rested on the black microscope stage and was protected by it from exposure to light. Examination and irradiation were begun. At this time the cells of Culture 582 appeared perfectly normal. The neutrophils were very active, and there was no sign of rounding up. *10 minutes later:* These cells were still very active; if any change had occurred, there was a slight stimulation. *30 minutes:* All of the neutrophils of Culture 582 were rounded up; their nuclei were slightly swollen, and their cytoplasm had coagulated, while their granules appeared somewhat hazy. At this time Culture 583 was given its first examination. The cells of this culture were perfectly normal; the neutrophils were moving around actively. No sign of the degeneration noted in Culture 582 could be seen.

The results shown by the cultures examined in this experiment may be briefly summarized as follows.

1. Radiant energy of such intensities and wave-lengths as originated from the microscope light source, acting on these cultures for periods of time varying from 2 minutes to about 70 minutes, produced an extreme and unmistakable effect on the cultures.

2. This action may be said to have had three phases, as follows. (*a*) There was first a short latent period during which no effect was noted. This period lasted from 3 seconds to 30 minutes. (*b*) Following the latent period there was an increase in the activity of the cell; the cellular movements were more extensive and more rapid. There was also a great decrease in the viscosity of the cytoplasm, as indicated by the tremendously increased amplitude of brownian movement of the granules within the cell. (*c*) Following this, there was a terminal period during which the cell rounded up. The viscosity of its cytoplasm still further decreased. This was accompanied by a swelling of the cell, or often, by coagulation.

3. The degeneration noted in the cells of this series of cultures, apparently coincided in every respect with the degeneration noted in previous cultures and described as of unknown etiology.

4. The continued action of light was not essential for the completion of the degenerative process in the cell. A very short exposure (3

minutes), produced little or no visible effect at the time, but even this was often found to be sufficient to cause the death of the cells in the culture within a few hours.

5. There was a curious irregularity in the time required for the degeneration of different cultures from the same series even when all were irradiated by the same source of light. For instance, the degeneration time of one culture was 35 minutes, while that of another in the same series was 90 minutes.

The positive findings summarized above have been confirmed by several hundred other cultures, examined at a later time in the accumulation of other data concerning this degeneration.

It having been definitely determined that irradiation of the cultures did cause a rapid degeneration of the white blood cells, an attempt was made to determine what wave-lengths of light caused this degeneration. The degeneration had been constant in its appearance upon exposure of the culture to light from any of the previously described sources. As a standard source of light for the further analysis of the problem, the 6 volt, 108 watt microscope lamp was chosen (Spectrum 3, Fig. 4). Besides the condenser system, 2 mm. of Corning daylight glass and a 35 mm. water-cell were also inserted in the path of the light. The light reaching the eye with this arrangement was of nearly daylight quality and of an intensity comfortable for visual work with a binocular microscope fitted with an oil immersion lens and 12 × oculars.

With this light source, and optical system, the degeneration might conceivably have been due to a concentration of infra-red rays on the culture, with a resultant heat coagulation of the cells. In order to investigate this possibility, a special glass "heat filter" (Spencer Lens Company) absorbing a large part of the infra-red rays (65–75 per cent) was also inserted into the optical path (Spectrum 4, Fig. 4).

In spite of the removal of such a large part of the infra-red heat waves from the light reaching the culture, the degeneration produced was apparently exactly the same as if no heat filter or water-cell had been used. While it is conceivable that this degeneration might have resulted from the residual infra-red waves reaching the culture through the optical system, the fact that there was no change in the rate of degeneration of the cells after the elimination of most of these

infra-red wave-lengths, makes it appear conclusive that the effect produced was not due to a heat coagulation of the cell.

With this possibility eliminated, the next step was to determine what range of wave-lengths was responsible for the degeneration. In order to do this the same light source and optical system were used, while various Wratten light filters were substituted for the Corning daylight glass. By this means the spectral range of the light admitted to the cultures was divided into several wave-length zones. The filters employed, together with their transmission zones and total transmissions (3), are listed in Table I, while spectrograms showing their light transmissions are shown in Spectra 5, 6, 7, Fig. 4.

TABLE I.

Filter		Wave-length zones in $\mu\mu$	Total transmission
Color of transmission	Number		
			per cent
Blue	45	320 to 390; 430 to 550; infra-red. (Spectrum 5, Fig. 4.)	5.0
Green	58	475 to 630; 690 to infra-red. (Spectrum 6, Fig. 4.)	23.0
Red	29	600 to infra-red. (Spectrum 7, Fig. 4.)	6.6

The results of this experiment showed that light transmitted by any one of these filters was active in causing the degeneration noted. Further, it was found that the time periods required for the degeneration of different cultures varied over a wide range for any one filter, and that the time periods required for the degeneration of different cultures of the same series, varied through the same wide limits when any one of the three filters, or when no filter was employed.

Hanging Drop Cultures of Whole Blood.

While the preceding experiments have definitely shown that the action of radiant energy did cause an extreme degeneration of the leucocytes, it was recognized that cells in this type of culture were under very abnormal conditions. They had been subjected to

centrifugation and lowering of temperature to 22°C. for about an hour, then washed with an artificial saline solution, and planted in an artificial culture medium. Any one or all of these manipulations might make these cells particularly susceptible to the action of radiant energy, either by increasing the fragility of the cells, or by otherwise changing their physical or chemical characteristics. In order to control as many of these factors as possible, another group of cultures was prepared and examined. In the preparation of these cultures the following technique was employed.

A drop of blood was drawn from a vein of a rabbit's ear, a cover-slip quickly touched to it, and then given a slight jerk so that the drop was spread out into a streak on the cover-slip. The cover-slip was then inverted on a hollow ground slide, and the edges of the cover-slip were sealed with "Salvoline." The culture was immediately placed at blood temperature and irradiation was begun within 10 minutes. These cultures were prepared in groups of three or four. Of these, one was irradiated while the other two or three were kept in the dark as controls.

The protocol of a representative culture of this series, together with its control, is presented below.

Culture 1062.—Examination and irradiation begun. The neutrophils at this time were moving around actively; the other cells appeared normal. *30 minutes later:* The neutrophils were moving around very rapidly as though markedly stimulated. *67 minutes:* The neutrophils had practically ceased moving around and were rounding up. *100 minutes:* The neutrophils within the illuminated area of the culture had all coagulated.

Culture 1063.—Control culture, kept in the dark. *200 minutes after inoculation:* Examination begun: The neutrophils were normal and were moving around fairly rapidly.

As may be seen from this experiment, the degeneration observed in saline and serum cultures of white blood cells appeared in a perfectly characteristic manner in these hanging drop preparations of whole blood. Since this was true, it appeared certain that the degeneration of leucocytes *in vitro* under the action of light, was not dependent upon prolonged temperature changes, the centrifugation to which the cells in culture were subjected, or the relatively rough handling necessary in the preparation of the tissue cultures, nor was it dependent on changes in saline concentration to which the cells in culture were subjected. It seemed to be a property of the leucocyte as it occurred in freshly drawn blood.

This degeneration of the leucocytes in hanging drop cultures of whole blood was also found to occur in perfectly typical manner upon irradiation of the culture through the various light filters previously described. It was also found to occur as usual upon irradiation of the culture through a 6 mm. thickness of Corning "Noviol C" glass, which transmitted only wave-lengths longer than 480$\mu\mu$, and a heat filter made up of a 3 cm. thickness of 5 per cent cupric sulfate.

In examining the data accumulated in all of these experiments, however, a tabulation of the time required for the degeneration of

TABLE II.

Irradiation Time Required to Cause Polymorphonuclear Neutrophils to Round Up, in Hanging Drop Cultures and in Hanging Drops of Whole Blood.

In tissue culture		In hanging drops of whole blood	
Culture number	Time	Culture number	Time
	min.		*min.*
472	10	1051	170
507	10	1059	95
587	30	1060	45
588	35	1062	67
589	90	1317	120
608	60	1320	58
612	12	1322	75
614	41	1512	60
618	7	1577	55
631	14	1578	60
Average.....	31		80

the leucocyte, *e.g.* the neutrophil, in ten representative hanging drop preparations of whole blood, and in ten representative tissue cultures prepared from the buffy coat of the centrifugated clot, showed that in the former the average time was 80 minutes, in the latter, only 31 minutes. Such a comparison of the degeneration time of representative cultures of both types is presented in Table II.

DISCUSSION.

From the data presented, it may be seen that the degeneration observed in these cultures of blood cells may be ascribed to the action

of light on the cells. It is well known that the Röntgen or ultra-violet regions of the spectrum may have a very profound effect on the organism, and the present work indicates that, certainly in the case of the blood cells *in vitro*, this action extends far down into the visual spectrum, certainly lower than 600 double micra. Further, as in the case of the generally known action of the shorter light waves of the spectrum, the action of these longer light waves is to produce first a stimulation, and later a degeneration of the irradiated cells. That this process of degeneration was conditioned by more than one factor is indicated by the divergent courses which were sometimes seen in the degeneration of different cells of the same type. In different cultures, cells were seen in which there was little swelling, but a coagulation of the cytoplasm. In other cultures the cells died in an expanded or lobulated condition (Fig. 3), while in still others, cells were seen with peculiar pouchings of the nuclear or cell walls which gave evidence that at least one change in structure had been a weakening of these walls. Nor were these so much the peculiarities of single cells, for generally the cells in any one culture, and occasionally, in any one series of cultures, showed a curious similarity in their degeneration. In emphasizing these divergent types of degeneration, however, it should be remembered that in the great majority of cultures there was a remarkable uniformity in the process (Fig. 1).

There were two terminations commonly seen in the degeneration of the neutrophil, under the influence of light. In the one, the cell swelled to about three times its normal diameter, sometimes even extruding some of its contents; whereas, in the other process, there was more of a gradual coagulation of the cell. What the fundamental differences are, which decide the type of degeneration any one neutrophil will undergo, has not been determined.

The final achromatic dead cells resulting from neutrophils which had undergone the coagulative type of terminal degeneration were often indistinguishable from the typical "non-motile" cell described by Sabin (8). On the other hand, neutrophils which had undergone the hydropsical type of degeneration, produced dead cells which were tremendously swollen. When the swelling became so great as to rupture the cell however, the collapsed cell often looked like a typical non-motile.

There is a marked similarity between the hydropsical type of degeneration described above, and the type of degeneration described by Kubie and Schultz (4, page 101) for the neutrophil occurring in cerebrospinal fluid. The technique given by these authors raises the question as to whether this degeneration noted by them was not due, in part at least, to the action of light during the handling of the cells. The similarity between the degeneration that they describe and that shown by the neutrophils in the present study may readily be seen by a comparison of their Figs. 11*b* and 12*b* with the present Fig. 1.

As was pointed out in the descriptions of the degeneration of the different types of leucocytes under the action of light, one very striking difference between the cells is the amount of fatty material which becomes visible within their cytoplasm during their processes of degeneration. The cell liberating the greatest quantity of this fatty material was the monocyte, while the neutrophil liberated the next greatest quantity. All other cells liberated relatively little, and none has been observed in the lymphocyte. The fundamental causes of this difference have not been indicated.

We are at present unable to explain the difference in the time required for the degeneration of the leucocytes in serum cultures and in hanging drop cultures of whole blood. It is conceivable, however, that although the rougher handling and abnormal solutions in the cultures planted in serum, may not be the factors sensitizing the leucocytes to light, they may, by some increase in the cell fragility, for instance, make the leucocyte more easily killed upon irradiation. Or it is possible that the longer time required for the degeneration of the leucocytes in hanging drops of blood may be solely due to the presence of red blood cells, and this action may be due either to their reducing the actual amount of light reaching the leucocyte, or, more probably, to other physical or chemical properties which the red cell may possess.

In considering what relation this degeneration of leucocytes *in vitro*, under the action of light, may bear to the action of light on these cells *in vivo*, the conditions in the two instances are so dissimilar that comparison is difficult with our present data. *In vitro* the cells are manifestly under artificial conditions; *in vivo* experimental results are

complicated by systemic reactions. It is significant, however, that in the work of Reed (7), and also of Clark (2), with light from the unfiltered iron arc, irradiation resulted in an immediate drop in the total white cell count. It is at least possible that in each of these instances irradiation may have resulted in injury to the leucocytes *in vivo* and their subsequent removal from the blood stream by systemic agencies. That such injury did occur in the work of Miles and Laurens (6) is positively indicated by the fact that these authors report that following irradiation, in addition to marked fluctuations in the white cell count, there were many "smudges" in the blood smears, these indicating a rapid destruction of white cells.

It should be noted however, that upon irradiating animals with light from the iron arc, filtered through glass, Clark found no such drop in the total count as she had found to follow irradiation with unfiltered light. There was, however, a marked lymphopenia, compensated by an increase in the number of neutrophils. This source of light, filtered through glass, might be considered as fairly comparable to the source used in the experiments described in this paper, whereas the light used by Reed, by Miles and Laurens, and by Clark, in her experiment with unfiltered light, was very rich in ultra-violet wave-lengths. As a consequence of these discrepancies, the nature of the relationship between the results reported in this paper and those reported by the above workers, is far from clear.

SUMMARY.

1. An extreme and rapid degeneration which occurred in tissue cultures of leucocytes from the blood of cats, guinea pigs, and rabbits, is described in detail.

2. This degeneration was found to appear in the culture when the cells were planted in any of the culture media tried, some of which were autogenous heparin plasma, autogenous plasma, autogenous serum, Tyrode solution, and mixtures of these with embryo juice.

3. The specific cellular changes which occurred are described for the different leucocytes. In general, there was first a latent period during which no change could be observed in the cell. Following this there was a period of stimulation during which the motion of the cell was greatly accelerated. This second stage has been observed

in all cells except the lymphocyte, in which it may possibly occur to a slight degree. Finally there was the terminal stage, the stage of degeneration, in which the cell rounded up, lost its motility, and either became badly swollen or else underwent a more or less complete coagulation.

4. The factor causing this degeneration was found to be exposure of the culture to light, as, for example, during microscopic examination.

5. By a reduction of the infra-red part of the spectrum, it was indicated that the effect was not due to a heat coagulation of the cells.

6. This degeneration was also found to occur in the complete absence of ultra-violet wave-lengths.

7. Further, it was shown that this degeneration was caused by light which lay within each of the three wave-length zones (1) 430$\mu\mu$ to 550$\mu\mu$; infra-red; (2) 475$\mu\mu$ to 630$\mu\mu$; 690$\mu\mu$ to infra-red; (3) 600$\mu\mu$ to infra-red.

8. No indication was given as to whether all regions of these zones were active in causing the degeneration, or whether the active rays are limited to certain wave-length bands lying within these zones.

9. This degeneration of the leucocytes under the action of light was also found to occur upon irradiation of hanging drops of whole blood. This is interpreted as showing conclusively that the degeneration was not dependent upon the additional factors of centrifugation, continued lowering of temperature, or the presence of abnormal saline solution.

10. It was noted, however, that the leucocytes in hanging drop cultures required a markedly longer time for their degeneration under the action of light than did the leucocytes in cultures prepared from the buffy coat and inoculated in serum. This is considered as possibly due, either to injury to the cell during centrifugation and subsequent handling, or to some action of the red blood cells present in large amounts in the hanging drops of whole blood.

11. In these hanging drop cultures of whole blood degeneration of the leucocytes was also found to occur when the light reaching the culture was first freed from the larger part of its infra-red and from all of its ultra-violet.

12. It was also shown that the same degeneration was produced by wave-lengths of light lying within each of the three wave-length zones defined in Section 6 of this summary.

I wish to express my appreciation to Dr. R. S. Cunningham, of the Department of Anatomy, under whose supervision this work has been carried on, to Drs. W. E. Garrey, and C. E. King, of the Department of Physiology, and to Dr. G. E. Cullen, of the Department of Biochemistry, for their helpful suggestions and aid. Further, I wish to thank Dr. Brian O'Brien, of Perrysburg, N. Y., who very kindly furnished me with the spectrographs printed in Fig. 4.

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EXPLANATION OF PLATES.

PLATE 9.

FIG. 1. Polymorphonuclear neutrophils of cat; from serum culture. × 1000. These cells show a typical degeneration resulting from an 80 minute exposure to light from the 6 volt 108 watt lamp described in the text, the light passing through an aspheric condenser of 6 cm. diameter, 35 mm. of water, and the microscope substage condenser (glass). By a comparison with the eosinophils shown in Fig. 2, which are photographed at the same magnification, and which are only slightly swollen, it may be seen that these neutrophils are swollen to over three

times their normal diameter. The spherical nuclei and oil droplets are easily seen.

FIG. 2. Polymorphonuclear eosinophils of cat; from serum culture. × 1000. Irradiated for 30 minutes by light from the same light source. Note the swollen nuclei and the tendency for the nuclei to become spherical. The cells are swollen to about one and one-fourth times their natural size.

FIG. 3. Polymorphonuclear neutrophils of rabbit; from hanging drop of whole blood. × 1000. Irradiated for 4 hours with the above light source, the light also passing through 2 mm. of Corning daylight glass, 20 mm. of plate glass, and the infra-red filter described in the text. Note that in this slide the cells have coagulated and died in a partially expanded condition, and that they show little swelling. These cells showed little change in the 2 hours preceding photographing.

PLATE 10.

FIG. 4. Spectra of different light sources used for the irradiation of cells. (1) Spectrum of mercury arc, for comparison. (2) 60 watt, 110 volt, tungsten filament, vacuum-filled, electric globe. Light passing through 35 mm. water, 250 mm. air, glass substage condenser. (3) 108 watt, 6 volt, tungsten filament, gas-filled electric globe. Light passing through a 6 cm. aspheric glass condenser about 1 cm. thick. (4) As in 3, except that light also passed through 35 mm. of water, the special heat filter described in the text, 20 mm. lime-soda glass, 2 mm Corning daylight glass, and the microscope substage condenser (glass). (5) As in 4, except that Wratten Filter 45 was substituted for the daylight glass. (6) As in 4, except that Wratten Filter 58 was substituted for the daylight glass. (7) As in 4, except that Wratten Filter 29 was substituted for the daylight glass.

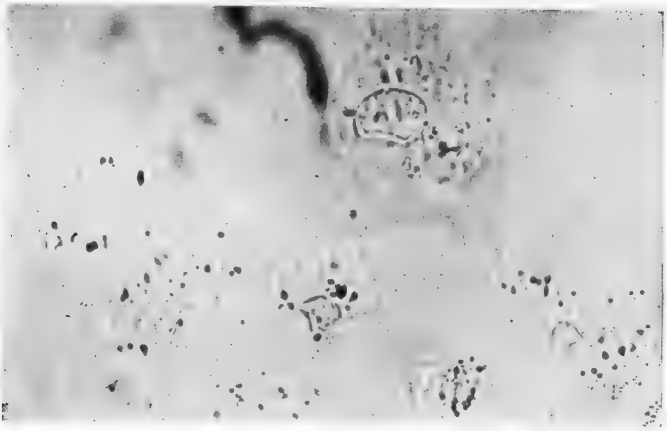

FIG. 1.

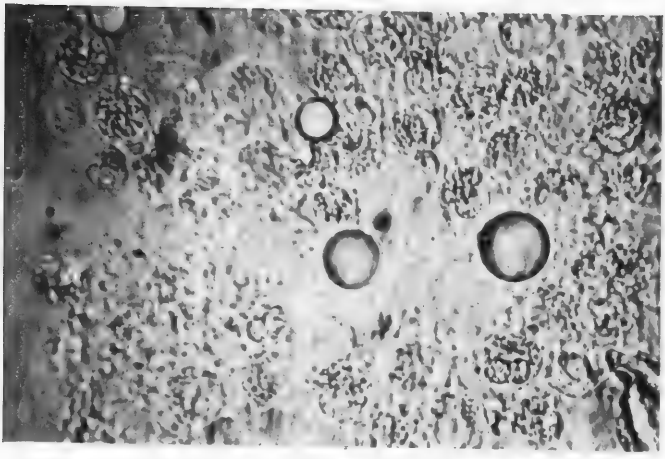

FIG. 2.

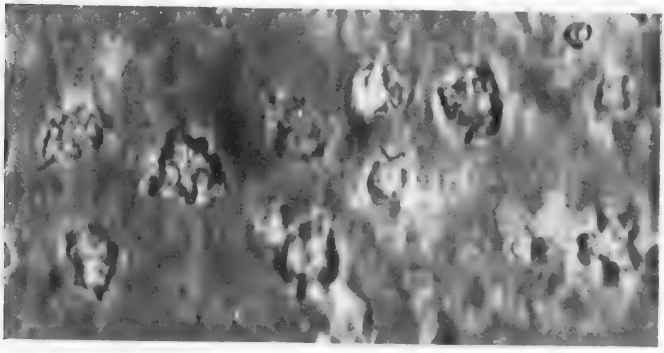

FIG. 3.

(Earle: Effect of light on blood and tissue cells. I.)

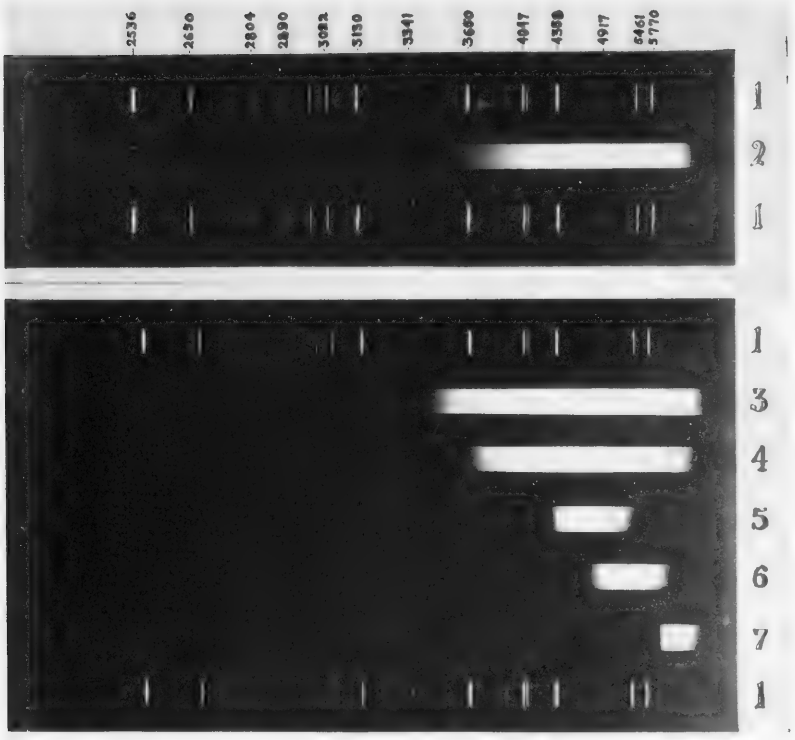

FIG. 4.

(Earle: Effect of light on blood and tissue cells. I.)

THE TRANSFER OF RAT ANEMIA TO NORMAL ANIMALS.

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PLATE 11.

(Received for publication, June 16, 1928.)

It has previously been shown by Lauda (1) that following extirpation of the spleen rats develop a severe anemia, characterized by destruction of red blood cells, hemoglobinurea, leucocytosis and an increase of blood platelets. This condition Lauda regarded as infectious and due to some type of virus, which might be vegetating on the mucous membrane of the stomach or intestine and be held in abeyance by the spleen. When the spleen is removed the virus gains entrance into the body and after an incubation period of a few days attacks the blood corpuscles and produces the anemia, the leucocytosis being a response to the infection. Lauda was unable to reproduce this anemia by the transfer of blood or organs to normal animals (except in one instance) but was able to produce it in rats from which the spleen had been removed and which had not yet developed the anemia or had recovered from it. Lauda's observations on rat anemia were subsequently confirmed by Mayer (2) and his associates who further noted that the red blood corpuscles of the anemic splenectomized rat contain small bacilliform bodies which resemble the bodies previously seen by Mayer (3) in the blood of rats with experimental trypanosomiasis and treated with Bayer 205. These bodies Mayer had named *Bartonella muris ratti* because of their resemblance to *Bartonella bacilliformis*, the etiological agent of Oroya fever. Mayer was unable to produce anemia in normal rats with blood containing *Bartonella muris* from anemic rats or to demonstrate *Bartonella muris* in the blood of the injected normal animals. Mayer (4) then observed that *Bartonella muris* could be eliminated from the blood of splenectomized rats by treatment with various compounds of arsenic. Injections of bartonella-containing blood produce anemia in such treated animals, with a multiplication of the bodies on the red corpuscles. Blood without the bartonella fails to produce the anemia. On the basis of these transfers Mayer concluded that *Bartonella muris ratti* is a living organism and the etiological agent in rat anemia after splenectomy.

The presence of *Bartonella muris ratti* in the blood of anemic splenectomized rats has subsequently been confirmed by Lauda and Marcus (5) and similar findings have been reported by Bayon (6) in England, de Faria and Cruz (7) in South America and by Jaffé and Willis (8), Noguchi (9) and Cannon, Taliaferro and

Dragstedt (10) in North America. Bodies resembling *Bartonella muris ratti* have been found in the blood of other species of normal animals and named *Grahamella*, but whether they are living organisms and have any relation to pathologic conditions has not yet been determined. *Bartonella muris ratti* has occasionally been observed in normal rats, notably by Lauda (5) and by Jaffé (8).

Bartonella-like organisms from rats have been cultivated for brief periods on artificial media by several observers, notably Mayer (11), Bayon (6) and Noguchi (9), but none of these cultivated organisms have been identified as the cause of the anemia owing to the lack of susceptible animals upon which to test them.

As a result of these investigations it may be regarded as established that in many strains of tame and wild rats splenectomy is followed by anemia and that this anemic blood always shows *Bartonella muris ratti* on the red cells. Thus far the anemia has not been reproduced in normal animals (*i.e.* those still possessing the spleen) nor has *Bartonella muris* been transferred successfully to normal animals.

During the past 2 years we have splenectomized a series of 66 rats with the following problems in mind:

1. To determine whether white rats in Baltimore develop anemia after splenectomy with *Bartonella muris ratti* on the red blood corpuscles.
2. To find some normal animal in which anemia can be produced by transfer of material (blood or organs) from anemic splenectomized rats.
3. To cultivate *Bartonella muris ratti* on artificial media.
4. To determine the relationship of *Bartonella muris ratti* to anemia.

A. The Anemia of Splenectomized Rats.

In two strains of rats available for this work, removal of the spleen is followed by an anemia like that described by Lauda and *Bartonella muris ratti* appears in the blood.

One is a strain of white rats purchased from a Baltimore dealer, the other a strain of hooded rats raised in the School of Hygiene. In a series of 58 rats some grade of anemia has appeared in all instances. In some cases the red cell count drops to between 2 and 3 million cells per c. mm. from an original count of 10–11 millions. The hemoglobin drops to 20–30 per cent (estimated by the Sahli hemoglobinometer) and hemoglobin appears in the urine. The white blood corpuscles rise markedly, the count increasing from an original count of about 16,000 to perhaps 35,000 per c. mm., and sometimes to 65 or 70,000. The increase is

largest in the polymorphonuclear neutrophils but the mononuclears also show a relative and absolute leucocytosis. At the same time there is apparently an increase of blood platelets, anisocytosis is often marked, there is polychromatophilia and a shower of normoblasts follows the destruction of red cells. The phagocytosis of red blood cells by the circulating mononuclears is characteristic. There may be as many as a dozen red cells in a single mononuclear. In all instances the blood of these anemic splenectomized rats contains *Bartonella muris ratti*. These structures appear on the 2nd to the 3rd day after splenectomy and their number corresponds to the grade of the anemia which subsequently develops.

Of the 58 rats splenectomized in the first series all developed bartonella anemia and 12 died of anemia from 7–12 days following the operation. 17 others died following bleeding from the heart for transfer of the virus to other animals. All of these showed a rapidly falling hemoglobin and many bartonellas on the red cells, and gave every promise of developing a severe and perhaps fatal anemia. 30 rats recovered from the anemia completely and showed a normal blood picture within about 4 weeks. 8 of these underwent a spontaneous or induced relapse 4–8 weeks later and in every case died of the anemia.

In addition 8 Littlestown rats were splenectomized which did not develop anemia nor show bartonellas except after exposure to infected rats.

The following protocol shows the blood changes in a splenectomized hooded rat which succumbed to the anemia.

Protocol for Rat 21.

Hooded Rat (School of Hygiene Strain).

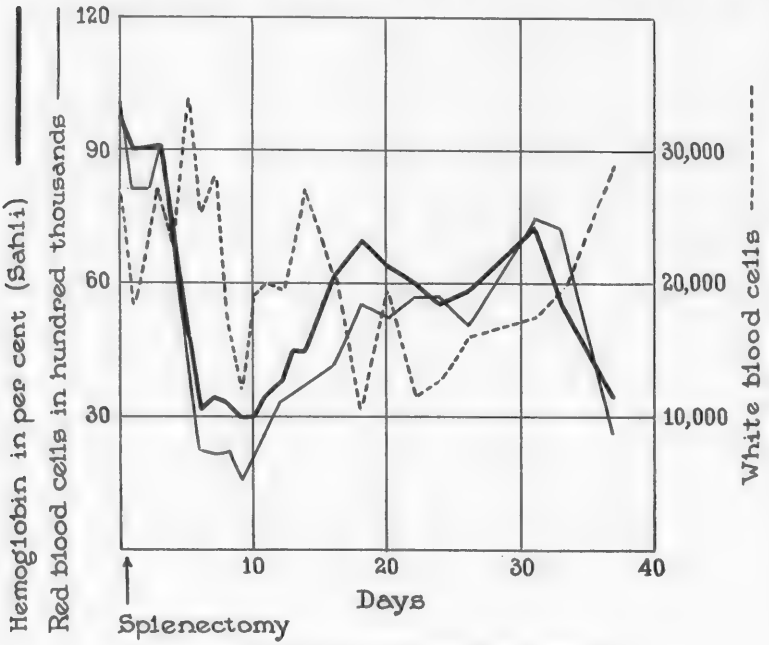
Date	Hemoglobin	Red cells	White cells	Observations and operations
Jan. 7	93	10,730,000	15,000	
" 9	91	10,770,000		Splenectomized
" 10	80	8,780,000	15,800	
" 11	82	9,760,000	17,100	Occasional rods
" 12	80	9,870,000	15,400	Few rods
" 13	75	8,740,000	20,900	Many "
" 14	43	5,040,000	30,200	" "
" 15	21	2,160,000	19,700	" " polychromatophilia, anisocytosis
" 16	22	2,220,000	14,700	Fewer " " "
" 17	27	2,110,000	7,450	" "
" 18	27	2,690,000	6,950	" "
" 19				Found dead

Text-fig. 1 shows the characteristic blood changes in a splenectomized rat which developed a severe anemia, recovered and subsequently had a relapse.

B. Production of Anemia in Normal Animals.

Transfer to Young Rats.

Intraperitoneal or intravenous transfer of blood from anemic splenectomized rats has been without effect on normal adult rats (with certain exceptions to be noted later), normal adult guinea pigs,



TEXT-FIG. 1. Rat 22. Splenectomized January 9.

normal adult rabbits and half grown dogs. Anemia did not develop after the transfers nor did the blood show *Bartonella muris*. Intraperitoneal injection of young rats about 3 weeks old, however, gave very different results. The blood of anemic splenectomized rats, taken 3–6 days after removal of the spleen, will produce anemia in young normal rats with intact spleen, provided that these rats are not too old or too large.

Rats 20–30 gm. in weight give the best takes, rats 40 gm. in weight are usually less satisfactory and with 60 gm. rats inoculation is frequently without effect. The disease develops in these young normal animals in much the same way that it does in adult rats after splenectomy. *Bartonella muris* appears in the blood in 1–3 days and the blood count begins to drop. On the 4th or 5th day the count may reach a minimum of about 3,000,000 cells per c. mm. The hemoglobin drops correspondingly, often to 30–40 per cent from 70–80 per cent and hemoblogin may appear in the urine. The leucocyte count does not change. In normal young rats this count is usually about 8,000 per c. mm. and the count is not appreciably increased at any time after inoculation with anemic blood, nor is there any definite increase of blood platelets. This failure of increase of leucocytes and of blood platelets is the only difference which has been noted between the blood picture in the adult anemic splenectomized rats and the young rats which develop anemia after transfer of bartonella-containing blood. In severe cases the animals may die in 5–8 days and at autopsy the mucous membranes and the organs are very pale. There is some icterus of the skin, the liver looks fatty and hemoglobin may be present in the urine in the bladder. The spleen is often notably increased in size.

The following protocols show the effect of the inoculation of normal animals with blood from anemic splenectomized animals.

Protocol of Rat N143.

Young normal rat weighing 35 gm. Injected April 17 intraperitoneally with 0.3 cc. whole blood from Rat N142 (third transfer from original splenectomized Rat 57).

Date	Hemoglobin	Red cells	White cells	Operation and observations
Apr. 17	70	7,780,000	8,500	Injected 0.3 cc. blood
" 18	62	6,490,000	8,600	Occasional rod
" 19	54	5,525,000	8,370	Few rods
" 20	52	4,160,000	9,300	Rods, polychromatophilia, anisocytosis
" 21	44	3,850,000		Occasional rod polychromatophilia, anisocytosis
" 24	64	5,720,000	8,250	Occasional rod

Protocol of Rat III 14–1.

Young rat, 35 gm., injected intraperitoneally with 0.5 cc. blood from splenectomized Rat 23.

Date	Weight	Hemoglobin	Red cells	White cells	Operations and observations
	gm.				
Jan. 16	35.0	75	7,000,000		Injection 0.5 cc. blood
" 17					Many rods
" 18	37.0	48	3,230,000		" "
" 19		32	2,620,000		Occasional rod
" 20	39.0	28	2,160,000		Rods
" 21		26	2,300,000	3,300	Few rods. Died few hours later

Autopsy.—Liver yellowish, spleen mottled yellow and red. Bladder filled with blood-tinged urine, slight icterus of skin. Cells in liver and spleen show phagocytosed red blood cells.

Success in the transfer of the virus of rat anemia to normal animals depends on a number of factors. The best results have been obtained with well nourished rats on a good balanced diet which reach a weight of 30–40 gm. in about 3 weeks. Ill nourished rats which are older at 30–40 gm. weight are not so satisfactory as younger rats. Rats over 60 gm. are about as resistant as normal adult rats, at times however, a few bartonellas may appear and a light anemia develop. Moreover, the virus in the original splenectomized rats varies greatly in its potency. In some instances it seems to have little effect on the normal animals while in other cases it may give excellent takes. Blood in the early stages of the anemia, 2–4 days after splenectomy, contains more potent virus than after the peak of the anemia is reached, and during or after recovery when bartonellas have disappeared from the circulation, the blood in our experience invariably loses its ability to produce anemia.

Blood from these normal animals in which anemia has thus been produced contains the virus of the disease. On transfer to other animals anemia develops and *Bartonella muris* appears in the blood. Blood for transfer gives the best takes when the red blood cells show the heaviest infection with bartonellas. This is usually from 2–5 days after injection. This passage of the virus can be kept up for succes-

sive generations of normal animals. Nine different strains of the rat anemia virus have been obtained from time to time and carried through at least three generations. Of these one was carried through five generations, one through six generations, two through seven generations, one through nine generations and one through thirty generations.

After several passages the virus tends to become less potent so that a more moderate anemia develops, usually a hemoglobin reading of 40–50 per cent being the lowest. The bartonellas are less numerous in the blood, that is, fewer cells are infected and there are fewer bodies in each infected cell. These are usually the large deeply staining pleomorphic forms that appear after active multiplication has ceased, and which seem to be a resting or resistant phase of the bartonella. The virus which we have carried through thirty generations went through several periods of this apparently inactive phase in which little or no anemia is produced. The bartonellas are carried along from transfer to transfer, undergo multiplication for a period of perhaps 24 hours and then recede promptly. By making several successive transfers at 24 hour intervals into younger and smaller rats we were able to bring this virus back into a state of active multiplication on two different occasions. Whether our more rapid transfer and choice of young rats were really the deciding factors in reviving the bartonella anemia is impossible to say. It is theoretically possible that the virus must go through some sexual or symplastic cycle outside of the blood stream at certain intervals, and so renew its vegetative energy. At all events its virulence and the morphology of *Bartonella muris ratti* seem to vary from time to time. The smallest injection with which we have produced a good take in a young rat has been 1/20 cc. of infected blood, but 1/100 cc. is sufficient to cause the appearance of bartonellas in the blood stream after 24 hours, and 1/200 cc. after 48 hours. These small injections, however, are apparently easily handled by the host. The bartonella is destroyed or its multiplication inhibited, and no anemia develops. Our usual infecting dose is 0.2–0.5 cc. injected intraperitoneally.

In all, 212 young rats have been injected with blood from other anemic rats. Of these, eight have died with a fatal anemia in 5–8 days, while fifteen others died, probably of anemia, but the terminal records were not complete enough to be absolutely convincing.

Forty-one died after bleeding from the heart for transfers. As in the cases of the splenectomized rats, the best and most promising cases were always selected for transfer, so that many of these, killed in the early stages of the disease, might have gone on to a fatal issue, had they not been killed. Thirty-nine of the young rats died from causes which could not be directly connected with the anemia.

Transfer to Young Rabbits.

In addition to young rats, young rabbits have also been found susceptible to the virus of rat anemia. On three occasions young rabbits about 3 weeks old have developed characteristic anemia after intravenous inoculation of $\frac{1}{2}$ to 1 cc. of bartonella-containing rat blood. The blood count dropped to about 3,000,000 from 6,000,000 and the hemoglobin to about 20 per cent (Sahli). Bartonellas appeared on the blood corpuscles in 5–12 days and the height of the anemia was reached 3–7 days later. In none of these animals did the infection proceed to a fatal issue. The bartonellas rapidly disappeared from the blood and the blood count returned to normal. There was a leucocytosis at the peak of the anemia.

Blood from these rabbits, containing *Bartonella muris*, again produced a characteristic bartonella anemia in young rats, showing that the virus can be passed through rabbits and not lose its potency for rats.

The following protocol shows the production of anemia in young rabbits.

Protocol Rabbit 17.

Young normal rabbit 3 weeks old. May 26, injected with 1 cc. blood intravenously from splenectomized Rat 17.

Date	Red cells	White cells	Operations and observations
May 26	5,672,000		Injected 1 cc. blood Rat 17
" 27			No rods
" 28			" "
" 30			" "
" 31	6,128,000	9,200	Occasional rod
June 1			" "
" 2			" "
" 3			" "
" 4			" "
" 7	4,616,000	9,600	Few rods, polychromatophilia
" 8	3,936,000	9,000	Many rods
" 9	3,448,000	7,600	" " anisocytosis
" 10	3,040,000	6,800	Fewer "
" 11			Few "
" 12	2,836,000	13,360	Occasional rod
" 13	4,712,000		
" 14	4,608,000	8,400	
" 18	5,280,000	21,400	

"Littlestown" Rats.

Another strain of rats purchased from a dealer in Pennsylvania and designated by us as the "Littlestown" strain, did not develop anemia nor show *Bartonella muris* after splenectomy. There are some rats, therefore, which do not harbor the virus of rat anemia and do not come down with the disease until subsequently infected either by artificial injection of virus or by some natural mode of transfer. This is similar to the experience of Jaffé and Cannon who find some strains of rats always infected and some never, or only occasionally so. We have thus far splenectomized five animals in this group, which when kept in isolation did not show *Bartonella muris* nor develop anemia even after several months. In three others, which had been kept in the animal house in the same room with infected rats for several

months, splenectomy was followed by anemia with the appearance of bartonellas in the blood. Such adult Littlestown rats which do not harbor the virus are susceptible to the virus upon injection. After inoculation of 1 cc. of blood from an anemic animal, a few bartonellas appear on the blood cells and a light anemia develops. This is never as severe as in the young rats, and rapidly regresses, the bartonellas disappearing and the blood count returning to normal.

Bartonella muris ratti in Wild Rats.

All of the white and hooded rats used for splenectomy or transfer have been examined for bartonellas before operation or injection and in only one instance have we seen the rods. This was a young rat of the Littlestown strain which had been in the laboratory 2 weeks. In all we have examined about 300 rats. Wild rats on the other hand have shown a high incidence of bartonellas on the circulating blood cells. Out of thirty-nine examined, ten showed bartonellas. The rods were never numerous, usually only one or two in about four oil immersion fields, in two cases several on one cell in nearly every field. The rat in this series showing the heaviest infection with bartonella had many trypanosomes in the blood and a heavy infection with leptospiras in the kidneys. This is in accord with Mayer's observation that rats infected with trypanosomes may show bartonellas.

C. Spontaneous Infection.

As mentioned above, in the two strains of School of Hygiene rats, removal of the spleen is always followed by the appearance of *Bartonella muris* and a more or less severe anemia. Another strain of white rats (Littlestown strain) obtained from a dealer in Pennsylvania and reared in a room remote from infected rats showed no bartonellas and did not develop anemia following splenectomy. Others of these rats splenectomized about a month after removal to the common animal room however, came down with anemia in the usual way. If the uninfected splenectomized rats now are put in a cage with infected splenectomized rats they begin to show bartonellas in the blood in 7–14 days and usually come down with a fatal anemia

a few days later. Similarly, convalescent splenectomized rats undergo relapses if placed with infected rats.

These observations indicate the highly contagious nature of the virus of rat anemia and point rather strongly to an insect vector. Cages and food cups are sterilized with steam twice weekly and care is taken not to mix food or water cups from cage to cage. The chances for transfer of the virus by direct contact with feces of infected animals or by biting are therefore remote. All of our rats are infested with *Polyplax spinulosa*. An occasional adult *Cimex lectularius* has been found around the cages and young forms have been picked from some of the rats. Experiments are under way to determine if these lice and bedbugs can transmit the virus to uninfected animals.

D. Attempts to Cultivate Bartonella muris ratti.

Repeated attempts have been made to cultivate *Bartonella muris ratti* on artificial media. For this purpose all the usual laboratory media have been utilized and in addition Noguchi's tissue media and leptospira media. Rat infusion agar from young normal animals was prepared and the reaction adjusted to pH 6.4, 7.0 and 7.4. Blood of rabbits, horses and rats has been used for enrichment. Thus far no successful cultivation has been effected and it seems very doubtful to us whether the organism has ever been grown artificially. Tissue cultures of blood or organs from infected animals have yielded no conclusive results.

E. Viability of the Virus.

Blood drawn from the heart of an infected rat into a glass syringe with heparin or sodium citrate remains infective for young rats for at least 2 hours. After 24 hours in the ice box the virus is definitely attenuated, ordinary doses giving only a light take with few bartonellas in the blood and little or no anemia. 24 hours at room temperature or 37°C. completely destroys the virus. The virus and the bartonellas are killed by heating the blood at 57°C. for ½ hour. Injection of this heated blood causes neither anemia nor the appearance of bartonellas.

Contrasted with the short life of the virus *in vitro*, is its apparent indefinite survival in the animal body. A young rat injected with bartonella blood and kept in isolation for several months following recovery from anemia, will upon splenectomy again come down with typical bartonella anemia. A rat from the uninfected strain kept in similar isolation shows neither bartonellas nor anemia following splenectomy. Rats from the non-infected strain injected with bartonella blood or merely exposed for a period of 2 weeks or so to infection, develop bartonella anemia upon subsequent splenectomy. Rats therefore become bartonella carriers, either after exposure or as a result of injection and apparently remain carriers indefinitely. Any rat harboring bartonellas will develop anemia following splenectomy even though it may have had and recovered from a typical case by injection.

F. Immunity.

These cases of anemia in splenectomized rats which have once had the disease, and the spontaneous and induced relapses in recovered splenectomized rats point to the failure of the immune mechanism in the absence of the spleen. Moreover, serum from a normal adult animal will not protect a splenectomized animal from invasion by bartonella and the development of anemia, nor will it protect the young injected animal from the disease.

Natural infection among the rats presumably is effected by minute doses, either through the bite of an insect or the ingestion of infected material. Such doses are easily handled by the immune mechanism, *Bartonella muris* does not multiply on the blood cells and no anemia develops. This accounts for the failure to find demonstrable bartonellas in normal white rats, although there may be occasional organisms present in the circulating blood at times. The relatively enormous doses of virus given young rats in our injections so overwhelm the immune mechanism that infection readily occurs and anemia develops. The younger the rat the more severe the disease, and the more likely to be fatal. Normal adult rats cannot be infected even with large doses. Removal of the spleen however is all that is necessary to precipitate the latent infection. Why the young spleen is

unable to protect the animal against invasion with this virus and the adult spleen is usually so completely effective, is a matter for future investigation. As mentioned before, however, the adult "Littlestown" rat which is normally not infected, can be made to undergo a light attack after injection which indicates that previous infection probably has some influence on the protective power of the spleen. At all events, it appears evident that the immunity developed in growing rats is primarily dependent on the spleen.

With this in mind it was considered important to see if the adult splenic tissue acted as a reservoir of inhibitory substances for the bartonella anemia. The spleen from a normal rat was ground in a mortar with a little physiological saline solution and allowed to stand for 1 hour with half the quantity of blood from an anemic splenectomized animal. ½ cc. of the mixture was then injected into a young rat and a control rat was injected with an equal amount of blood without splenic tissue. The experiment was repeated four times, and in no case could we find any demonstrable difference between the control animals and the rats with splenic tissue. Both developed bartonella and a light or moderate anemia according to the potency of the virus used.

Attempts to produce an immune serum in rabbits have so far proven unsuccessful. A rabbit which has once had bartonella anemia cannot be made to undergo a relapse by further injections. Serum from such an "immune" rabbit which has recovered from the anemia and been given two subsequent injections of bartonella blood fails to protect splenectomized rats from infection and anemia. This is similar to Noguchi's experience with *Bartonella bacilliformis*.

G. Nature of Bartonella muris ratti.

In view of our inability to cultivate *Bartonella muris*, some question may arise as to its character, whether it is really a bacterium or represents a stage in the life cycle of some protozoan parasite.

According to our observations *Bartonella muris ratti* is a small bacillary body about 0.1 μ in width and 0.5–1.0 μ in length, which appears to lie on the surface of the red cells (Fig. 1). Stained by Wright's blood stain it takes a bluish hue with

the suggestion of redder granules at the ends. Stained by Giemsa the whole rod has a redder hue. It is decolorized by Gram's method and takes the fuchsin counterstain very faintly. When the rods first appear they are thick, dark and short, often diplococcoid, occur singly and only on an occasional cell. These forms are followed by dumb-bell-shaped or granular rods and long slender bacillary bodies. More cells are infected and there may be two or four rods on a cell. As the infection progresses the rods appear shorter, usually in parallel rows and chains and the majority of the red cells contain from a few to several dozen. Any time from the 5th–10th day after splenectomy, the majority of the infected cells disappear rather suddenly from the blood stream, and the remaining infected cells reveal larger deeply staining, highly pleomorphic bodies, with occasional peculiar prolongations of the cytoplasm. There may also be ring forms, thicker and more deeply stained on one side. These forms, which seem to be related to the earlier ones, suggest a stage in the life cycle of some protozoan.

Occasionally groups of bartonellas are found free in the plasma, but these are usually associated with fragments of laked corpuscles. The bartonellas do not stain well when they are free from the corpuscles, hence it is more difficult to identify them with certainty, particularly to distinguish them from granules of broken platelets. The platelet granules differ from the rods however, by their greater thickness and less delicate outline and the redder hue with Wright's stain. The rods are also readily distinguished from the basophilic granules of the red cells, from chromatin dust and the Howell-Jolly bodies, all of which tend to increase during the course of anemia. The reticulated red cells also increase during the infection. By staining with cresyl blue and then by Wright's the difference between the bartonellas and reticulum is well brought out. The reticulum appears as bluish rods or masses of rods principally in the center of the cell, while the bartonella rods are distinctly redder and appear toward the periphery of the cell. Only a few reticulated cells are infected with bartonellas and then usually very lightly. We have not been able to see *Bartonella muris* in unstained smears or on living cells in the hanging drop. Fresh dark field preparations, however, reveal the bartonellas on the cells as non-motile rods. They apparently do not stain with neutral red. Many cells in bartonella anemic blood may show neutral red-stained rods, but from their position and distribution we believe that they are reticulum rather than bartonellas.

H. Relation of Bartonella muris ratti to Rat Anemia.

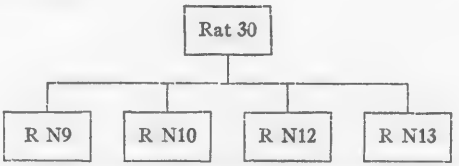
Since *Bartonella muris* has never been grown artifically, and rat anemia produced by cultures, it cannot be regarded definitely as the cause of the disease. There is, however, some collateral evidence which makes it highly probable that this organism stands in etiological relation to it. *Bartonella muris* is always associated with the anemia

and varies with the extent of blood destruction. If it appears on the corpuscles in large numbers or if many corpuscles harbor it the anemia is apt to be severe. When only a small number appear in the blood, the anemia is mild. Moreover, the bartonellas seem to be destroying the cells. In heavily infected blood one can find all stages of blood destruction, frequently broken down corpuscles and shadows of cells with bartonellas still clinging to them. Only blood containing *Bartonella muris* can produce the anemia. Blood in which bartonellas cannot be demonstrated has regularly failed to produce it. Red blood corpuscles harboring bartonellas, freed from serum and washed repeatedly in physiological saline, produce the anemia. The bartonellas can be demonstrated in stained films of these washed cells. The plasma removed from these same cells does not produce immediate anemia after injection. That a few bartonellas are actually present, however, is demonstrated by their appearance in small numbers in the injected rat after a prolonged incubation period and the subsequent production of a moderate anemia. Blood from rabbits in which a mild anemia has been caused by inoculation of bartonella rat blood and which harbors *Bartonella muris*, will produce a characteristic anemia in young rats which again show the bartonellas. As pointed out above, the virus producing the anemia and the bartonellas are both destroyed at 57°C. It can be said, therefore, that red blood corpuscles plus *Bartonella muris* produce anemia and that *Bartonella muris* either represents the virus or that the virus is attached to the corpuscles and *Bartonella muris* is an accompanying organism.

The following experiment illustrates the production of bartonella anemia in young rats by the injection of whole blood or washed cells and the failure of plasma to cause anemia except after a prolonged incubation period.

Experiment with Virus 30.

Rat 30 bled from heart 5 days after splenectomy. Rat N9 injected with 0.2 cc. and Rat N10 with 0.3 cc. whole blood. Blood centrifugalized. Rat N12 injected with 0.5 cc. plasma. Cells washed in physiological salt solution, resuspended and 0.5 cc. injected into Rat N13.



Weight	30 gm.	32 gm.	24 gm.	32 gm.
Injection	0.2 cc. whole blood	0.3 cc. whole blood	0.5 cc. plasma	0.5 cc. washed cells
Bartonella				
2 days	Many	Many	None	Many
10 days	Occasional	Few	Few	Occasional
Hemoglobin				
Before injection	75	77	80	72
1 day after injection	67	65	82	70
2 days " "	48	53	78	70
3 " " "	30	41	80	24
4 " " "	31	36	78	27
10 " " "	65	48	50	65

SUMMARY AND CONCLUSIONS.

Fifty-eight white and hooded rats have been splenectomized and all of them have shown a more or less severe anemia and an infection of the red blood cells with *Bartonella muris*. Another strain of white rats obtained from Littlestown showed no anemia and no bartonellas in the blood after splenectomy, until exposed to infected rats. Others of these Littlestown rats, kept in the laboratory for some time before operation and exposed to infected rats, came down with bartonella anemia within 6 days after splenectomy.

Whole blood or the washed red blood corpuscles from splenectomized rats which show bartonellas and anemia will produce a similar condition in young rats when injected intraperitoneally. Adult rats of

strains which harbor the virus (as demonstrated by splenectomy) cannot be infected by injection.

Intravenous inoculation of young normal rabbits with blood from an infected rat will sometimes produce a similar infection and anemia in the rabbit, and the virus can then be transferred back to young rats.

The virus of rat anemia may be transferred from young normal rat to young normal rat with the appearance of *Bartonella muris* and the production of anemia. In the early transfers the disease may be fatal, but it usually becomes milder in successive passages.

Although we have not yet been able to cultivate *Bartonella muris* and prove its etiological relationship to rat anemia by inoculation of cultures, we have added to the evidence that *Bartonella muris* is the cause of the anemia. Washed red blood corpuscles, containing bartonellas, will produce the anemia in the usual way while plasma from the same cells will either fail to produce it altogether or only after a prolonged incubation period. Blood heated to 57°C. for ½ hour fails to produce anemia or the appearance of bartonellas in the blood of inoculated animals.

From these observations the following conclusions may be drawn:

1. All rats which harbor *Bartonella muris ratti* come down with a more or less severe anemia after splenectomy.

2. Young rats which have not yet developed an immunity undergo the typical anemia after intraperitoneal injection of blood from a splenectomized animal in the early stages of the anemia.

3. Young rabbits may show bartonellas and develop anemia following intravenous inoculation of infected blood.

4. The virus of rat anemia and *Bartonella muris ratti* may be transferred from normal animal to normal animal for successive generations. Such strains have now been transferred for five, nine and thirty generations.

5. The resistance of rats to bartonella anemia is almost wholly dependent on the spleen. Other organs do not take over this function of protection as shown by the relapse of splenectomized rats many months after recovery. Young rats which have recovered from an attack of anemia are not protected by this previous infection from the invasion of the virus following splenectomy. Adult splenic tissue

mixed with infected blood before injection does not inhibit or neutralize the virus.

6. The virus of rat anemia is highly contagious and rats exposed to infection acquire it in some unknown way.

7. *Bartonella muris ratti* represents the virus of rat anemia or at least cannot be separated from the virus because:

(*a*) The anemia in splenectomized and injected animals is always preceded by the appearance of bartonellas and the grade of anemia is proportional to the degree of infection with bartonellas.

(*b*) Washed corpuscles containing bartonellas always produce anemia. Plasma either fails to do so, or produces a mild anemia after a long incubation period with a few bartonellas in the blood.

(*c*) The thermal death point of virus and bartonella is the same.

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EXPLANATION OF PLATE 11.

FIG. 1. *Bartonella muris ratti* in the blood from a rat with severe anemia, 5 days after splenectomy. Stained by Wright's stain.

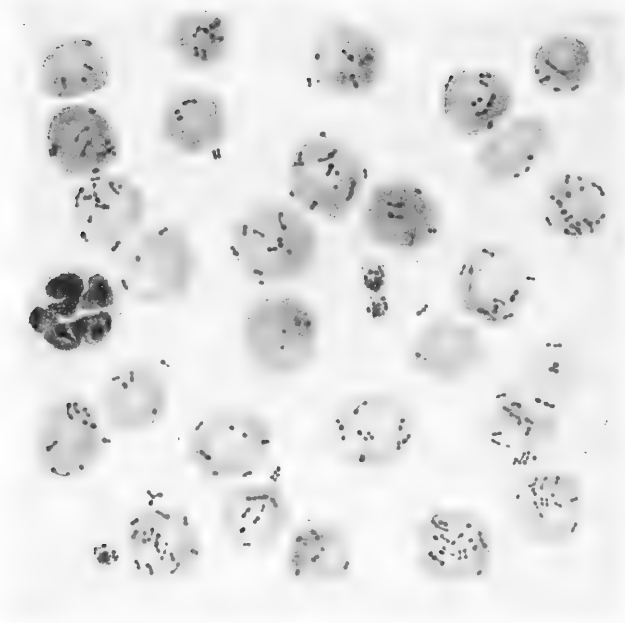

FIG. 1.

(Ford and Eliot: Transfer of rat anemia to normal animals.)

THE CONVERSION OF HEMOLYTIC STREPTOCOCCI TO NON-HEMOLYTIC FORMS.

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Bacterial variation has been studied by a number of workers and it is now a well established observation that avirulent variant cultures can be obtained from a number of microorganisms. Virulent cultures usually form smooth colonies on agar and avirulent variants are distinguishable by the rough appearance of their colonies. In the case of hemolytic streptococci, however, it has been shown (1) that these relations are reversed, that smooth colonies characterize an avirulent culture and that the rough form of colony indicates that a culture is either actually or potentially virulent. To avoid confusion, which might arise from this unusual relationship between virulence and colony form, the virulent type of colony has been designated the "matt" form and the avirulent type has been called the "glossy" form. A matt culture may be either virulent or attenuated but its virulence can always be enhanced by animal passage.

A number of matt strains of hemolytic streptococci were subjected to mouse passage; and in the course of these experiments two strains were encountered, both derived from cases of puerperal septicemia, which became progressively less hemolytic on blood agar as they were passed through a series of mice. One of these strains retained some hemolytic power after thirty mouse passages but the other strain (Henson) was completely deprived of its hemolytic properties by passage through five mice. At first the non-hemolytic passage culture was rejected on the theory that it arose from contamination in the peritoneal cavity but it was subsequently found that this strain could always be rendered non-hemolytic by passage through a few mice. Young colonies of the passage culture,

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which were opaque, greenish in color and non-hemolytic, did not affect the blood cells in the agar surrounding the colonies; but older colonies were surrounded by a narrow green zone. This non-hemolytic culture was passed through 74 mice and the peritoneal washings and hearts' blood of the mice were plated out daily on blood agar but the colonies remained completely non-hemolytic throughout the experiment. In contrast to this result the hemolytic character of the glossy variant of the same strain remained unaltered after passage through thirty mice.

The non-hemolytic culture was reverted to the hemolytic form by continued daily subcultivation on artificial media. Twenty transfers in broth caused the reappearance of a distinct halo of hemolysis around each colony but the degree of hemolysis remained subnormal until the culture had been transferred in broth 150 times. After this large number of transfers the culture was partially converted to the glossy state and two forms of colonies now appeared on blood plates, the matt form which was moderately hemolytic and the much more hemolytic glossy form. Broth cultures of the two forms were obtained by selecting suitable colonies and the passage of these two cultures through mice confirmed the previous experiments—the matt form became non-hemolytic but the glossy form retained its hemolytic properties.

It will be seen from the above description that three forms of the same strain, showing different degrees of hemolysis, were available:

1. The original matt culture was moderately hemolytic on blood agar and was avirulent for mice. (M.L.D. 0.5 cc.)

2. The glossy avirulent form, derived from the original culture by long continued subcultivation on artificial media, was much more hemolytic than the original form and its hemolytic properties were unaltered by mouse passage. (M.L.D. 0.5 cc.)

3. The non-hemolytic form, obtained from the original culture by mouse passage, was moderately virulent. (M.L.D. 0.00001 cc. after 47 passages.) Long continued subcultivation of this form caused it to revert to a mixture of the other two forms.

The Effect of Reduced Oxygen Tension on the Hemolytic Properties of Three Forms of Streptococcus hæmolyticus.

The effect of reduced oxygen tension on the three forms of culture was investigated. Colonies of the passage culture were completely non-hemolytic when freely exposed to oxygen on the surface of blood agar plates; but hemolysis became evident when the supply of oxygen was diminished by growing the culture in blood broth or in the depths of blood agar. When oxygen was more completely excluded, by cultivation in an anaerobic jar, the passage culture, and the original culture, both formed hemolytic colonies on the surface of blood agar

TABLE I.

Showing the Comparative Amount of Hemolysis, Which Surrounded Colonies of the Three Forms of Culture, under Different Degrees of Oxygenation.

	Surface colonies on blood agar plates in air	Surface colonies on anaerobic blood agar plates	Deep colonies in blood agar (aerobic incubation)
Original culture	++	++	++
Passage culture	0	+	+
Glossy variant	+++	+++	+++

0 = no hemolysis.

+++, ++, + represent the various degrees of hemolysis.

and the zones of hemolysis surrounding colonies of the two cultures were almost equal in circumference.

Table I gives the degree of hemolysis of the three forms of the same strain, on the surface of blood agar plates in air, on the surface of anaerobic plates and in the depths of blood agar.

Reed, Orr and Campbell (2) have shown that the filtrate from a culture of *B. welchii* has greater hemolytic activity on a suspension of reduced blood cells than on a similar suspension of oxygenated cells. The following experiment was done to determine whether the hemolytic activity of the passage culture of Strain Henson, which only appeared under anaerobic conditions, was due to the effect of diminished oxygen tension on bacterial metabolism or to the reduced state of the blood cells.

A 5 per cent suspension of rabbits' washed red blood cells in salt solution was divided into two parts, both of which were sealed with paraffin oil; one portion was reduced by passing hydrogen through the suspension for an hour and the other part was oxygenated by similar treatment with air. Hemolysin was prepared by filtering a culture of the original form of Strain Henson after 10 hours incubation; progressive dilutions of the filtrate in infusion broth were divided into two parts so that two sets of serial dilutions (volume 0.5 cc.) were obtained and, after all the tubes had been sealed with paraffin oil, 0.5 cc. volumes of reduced cells were

TABLE II.

Showing the Hemolytic Activity of a Filtrate from the Original Form of Strain Henson on Reduced and on Oxygenated Blood Cells.

Dilution of filtrates	Hemolytic activity of the filtrate on	
	Oxygenated cells	Reduced cells
1 in 2	++++	++++
1 in 4	++++	++++
1 in 8	++++	++++
1 in 16	+++	+++
1 in 32	+++	+++
1 in 64	+++	+++
1 in 128	++	++
1 in 256	+	+
1 in 512	+	+
1 in 1,024	−	−
Control	−	−

In all tables the following symbols are employed:

++++ = complete hemolysis.
+++ = almost complete hemolysis.
++ = incomplete hemolysis.
+ = slight hemolysis.
− = no hemolysis.

added to one set of dilutions with a capillary pipette and the same amounts of oxygenated cells were added to the other series. Readings were taken after the test had been incubated for 1 hour and the results, which are recorded in Table II, showed that the hemolysin was equally active on reduced cells and on oxygenated cells.

No steps were taken to exclude oxygen from the dilutions before the addition of the cells but, as the reduced cells retained their characteristic cherry-red color throughout the test, the conditions were considered satisfactory.

McLeod and Gordon (3) showed that peroxide is formed in broth cultures of pneumococci and Avery and Morgan (4) demonstrated that the quantity of peroxide formed depends on the surface area of broth exposed to the air. They found that strongly positive peroxide reactions could be obtained when a broth culture was incubated in a shallow layer allowing free access of oxygen, that deep broth cultures, with only a small surface area, produced smaller amounts of peroxide and that anaerobic cultures did not form any peroxide. They also

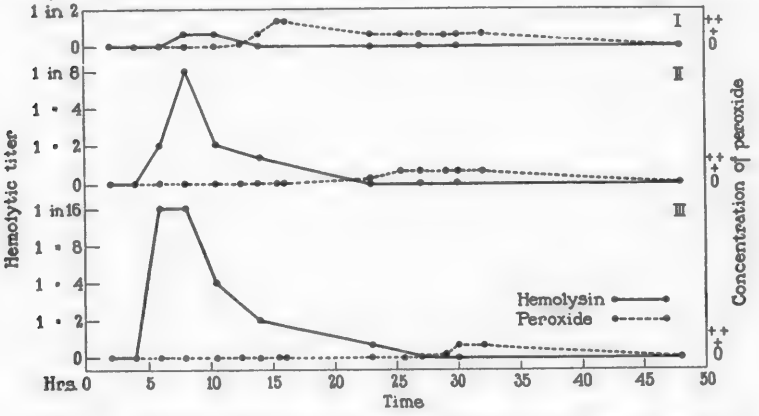

CHART 1. Showing the times at which hemolysin and peroxide appeared in three different cultures of the same strain of hemolytic streptococcus. I. Passage culture (non-hemolytic on aerobic blood plates). II. Matt attenuated culture (moderately hemolytic on aerobic blood plates). III. Glossy culture (very hemolytic on aerobic blood plates).

examined twenty-three strains of hemolytic streptococci and found that fifteen of these strains produced peroxide when grown in shallow layers of broth. The observations of Avery and Morgan run parallel to those already recorded in this paper—free exposure to air, a condition which is necessary for the accumulation of peroxide, prevented the formation of hemolysin by our passage strain of hemolytic streptococcus; conversely anaerobic conditions, which prevent the formation of peroxide, were necessary for the production of hemolysin.

The following experiment was done to see if any connection could be established between the formation of hemolysin and peroxide in shallow broth cultures of hemolytic streptococci freely exposed to air.

Three 2 liter Erlenmeyer flasks, each containing 50 cc. of infusion broth to which 20 per cent of normal horse serum had been added, were sown with 0.2 cc. of young cultures of the three forms of hemolytic streptococcus (Strain Henson) and placed in the incubator together. Samples of culture were removed from the three flasks at intervals and tested for peroxide by the potato-benzidine reaction; periodical titrations of hemolysin were also made by centrifuging samples of the cultures and testing the supernatants against a 5 per cent suspension of washed rabbit cells. The results of this experiment are given in Chart 1.

The original culture of Strain Henson (II on chart) formed a moderate amount of hemolysin which first appeared after 6 hours incubation and disappeared after 23 hours; the disappearance of hemolysin was immediately followed by the appearance of peroxide for the first time.

The passage culture of Strain Henson (I on chart), which was non-hemolytic, when freely exposed to oxygen on the surface of blood agar, produced less hemolysin in broth than either of the other cultures; and a comparison of the three curves brings out two points which demonstrate this relative weakness in hemolytic power, (1) the low hemolytic titer, and (2) the fact that hemolysin appeared later and disappeared earlier from this culture than from either of the other cultures. This deficiency in hemolysin production was associated with the ability to produce more peroxide than either of the other cultures; but it is interesting to note that peroxide and hemolysin did not appear in the culture at the same time and that the disappearance of hemolysin was immediately followed by the rapid accumulation of peroxide.

The supernatant fluid from a centrifuged culture of the glossy variant (III on chart) gave the highest titer and longest duration of hemolysin; and peroxide, which was absent until the hemolysin had vanished, did not appear until the culture had been incubated for 29 hours.

Each of the three curves shows that peroxide and hemolysin did not occur simultaneously in the same culture.

Avery and Neill (5) have shown that hemotoxin is inactivated in cultures of pneumococci by the oxidizing action of peroxide; and their observations, coupled with the result of the experiment described above and also with the observation that the passage culture of Strain Henson was non-hemolytic in air but hemolytic under anaerobic conditions, suggested that the disappearance of hemolysin from cultures of hemolytic streptococci was also due to oxidation.

Is the Rate of Disappearance of Hemolysin Increased by Oxygenation of the Medium?

The following experiments show that the disappearance of hemolysin from cultures of hemolytic streptococcus is not influenced by oxygenation.

Experiment A.—Two cultures of the original form of Strain Henson were grown in 10 cc. of infusion broth containing 20 per cent normal horse serum—in one case the culture was made in a test-tube so that only a small area of broth was in contact with the air; in the other case the same volume of broth was placed in a 250 cc. Erlenmeyer flask forming a very shallow layer with a large area exposed to the atmosphere. Hemolysin was titrated after 6 hours incubation and again after 24 hours incubation. Table III shows that the different degrees of aeration in these two cultures did not cause any difference either in the titer of hemolysin or in the rate of its disappearance.

TABLE III.

Showing that the Hemolytic Titer of a Culture of Streptococcus hæmolyticus is not Influenced by the Surface Area of Broth Exposed to the Air.

Dilution of supernatant	Hemolysin after 6 hrs. incubation		Hemolysin after 24 hrs. incubation	
	Shallow culture with large aerating surface area	Deep culture with small aerating surface area	Shallow culture with large aerating surface area	Deep culture with small aerating surface area
1 in 2	++++	++++	−	−
1 in 4	++++	++++	−	−
1 in 8	++++	++++	−	−
1 in 16	++	++	−	−
1 in 32	+	+	−	−

Experiment B.—Two cultures of the glossy form of Strain Henson were grown in shallow layers of broth in 250 cc. Erlenmeyer flasks; one flask was incubated in the air and the other in an anaerobic jar. Samples from the aerobic culture were tested for hemolysin at frequent intervals and as soon as the tests became negative the anaerobic jar was opened and the second culture was found to be free from hemolysin.

Experiment C.—A hemolytic filtrate, obtained from the glossy form of Strain Henson, was divided into two parts; one part was incubated anaerobically for 24 hours and the other part was incubated for the same time in air. Hemolysin had completely disappeared from both portions of the filtrate after 24 hours incubation.

The following experiments (D and E) were done to see if the addition of the reagent hydrogen peroxide to an actively hemolytic filtrate would reduce the hemolytic titer by oxidizing the hemolysin.

Experiment D.—Various quantities of hydrogen peroxide ranging from a dilution of 1 in 20 to a dilution of 1 in 2,000 were added to aliquot parts of a filtrate, prepared from a culture of the glossy variant of Strain Henson, and broth controls containing the same quantities of hydrogen peroxide were set up at the same time. After standing at room temperature for an hour the mixtures were tested for hemolysin and for peroxide.

TABLE IV.

Showing that the Hemolytic Titer of Supernatant Fluid from a Culture of Streptococcus hæmolyticus is Not Altered by the Addition of Hydrogen Peroxide.

Dilution of supernatant	Hemolytic titer of filtrate before addition of hydrogen peroxide	Hemolytic titer of filtrate 1 hr. after the addition of various quantities of hydrogen peroxide			
		Control. No H_2O_2 added to filtrate	H_2O_2 + filtrate 1 in 20	H_2O_2 + filtrate 1 in 200	H_2O_2 + filtrate 1 in 2,000
1 in 2	++++	++++	++++	++++	++++
1 in 4	++++	+++	+++	+++	+++
1 in 8	+++	++	++	++	++
1 in 16	+++	++	++	++	++
1 in 32	++	+	+	+	+
1 in 64	++	+	+	+	+
1 in 128	+	−	−	−	−
Control hemolytic test with broth to which the same quantities of H_2O_2 had been added.....		−	−	−	−
Peroxide test 1 hr. after the addition of various quantities of H_2O_2.....		−	++++	+++	−

It will be seen from Table IV that hydrogen peroxide did not alter the titer of hemolysin.

Experiment E.—The last experiment was repeated with the following modifications: Two 25 cc. volumes of hemolytic filtrate from the glossy variant of Strain Henson were placed in flasks and the same amount of broth was placed in a third flask. Equal quantities of hydrogen peroxide, making a dilution of 1 in 100, were added to one flask of filtrate and to the control broth. The three flasks, contain-

ing respectively, (1) untreated filtrate, (2) the same filtrate with the addition of hydrogen peroxide and (3) broth similarly treated with hydrogen peroxide, were then incubated at 37°C.; and the hemolytic titer of each specimen was tested at intervals of 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours and 23 hours. Peroxide tests were frequently made during the course of this experiment and the supply of peroxide was replenished on the two occasions when these tests gave negative results.

Periodical titrations demonstrated a gradual fall in the hemolytic power of the two filtrates but the rate of disappearance of hemolysin was not affected by the addition of peroxide as the falling titers ran parallel up to the 23rd hour when hemolysin had disappeared from both specimens.

Experiment F.—In this experiment, which again shows that peroxide does not destroy the hemolysin of hemolytic streptococci, a peroxide of bacterial origin was used instead of the reagent hydrogen peroxide. A culture of a microorganism, resembling *Micrococcus tetragenus*, which was known to produce large quantities of peroxide, was incubated in infusion broth containing 20 per cent of normal horse serum for 30 hours; it was then filtered and the filtrate, which gave a strongly positive peroxide reaction, was divided into two parts. The peroxide was removed from one portion of filtrate by adding a small quantity of washed blood cells; and, after removal of the blood by centrifuging, the filtrate which now gave a negative peroxide reaction, was used to control the original filtrate.

A hemolytic filtrate, prepared in the usual way from the glossy variant of Strain Henson, was used to make the following mixtures:

(1) 5 cc. hemolytic filtrate + 5 cc. broth.
(2) 5 cc. hemolytic filtrate + 5 cc. tetrad filtrate (bacterial peroxide).
(3) 5 cc. hemolytic filtrate + 5 cc. control tetrad filtrate (no peroxide).
(4) 5 cc. tetrad filtrate (bacterial peroxide) + 5 cc. broth.

The hemolytic titer and the peroxide content of these mixtures were determined immediately and a second series of observations was made after they had been incubated at 37°C. for 1 hour.

Table V which gives the result of the experiment shows that the hemolytic titer remained unaffected by the bacterial peroxide.

The results of the experiments described in this section appear to indicate that the disappearance of hemolysin from cultures of hemolytic streptococci is not due to oxidation.

The Influence of pH on the Disappearance of Hemolysin from Cultures of Hemolytic Streptococci.

It is an old observation that hemolytic streptococci form non-hemolytic colonies on the surface of blood agar plates which contain glucose; and the following experiments were undertaken to determine the influence of pH on the rate of disappearance of hemolysin from cultures of hemolytic streptococci in broth.

TABLE V.

Showing that the Hemolysin of Streptococcus hæmolyticus is not Destroyed by Bacterial Peroxide.

Dilution of mixtures	Hemolytic titer immediately after mixing hemolysin and organic peroxide				Hemolytic titer of the mixtures after incubation for 1 hr. at 37°C.			
	Hemolysin + broth	Hemolysin + tetrad filtrate (bacterial peroxide)	Hemolysin + control tetrad filtrate (no peroxide)	Tetrad filtrate (bacterial peroxide) + broth	Hemolysin + broth	Hemolysin + tetrad filtrate (bacterial peroxide)	Hemolysin + control tetrad filtrate (no peroxide)	Tetrad filtrate (bacterial peroxide) + broth
1 in 2	++++	++++	++++	−	++++	++++	++++	−
1 in 4	++++	++++	++++	−	++++	++++	++++	−
1 in 8	++++	++++	++++	−	+++	+++	+++	−
1 in 16	+++	+++	+++	−	+	+	+	−
1 in 32	+	+	+	−	−	−	−	−
1 in 64	−	−	−	−	−	−	−	−
Peroxide tests....	−	++	−	++	−	−	−	−

Two flasks, containing respectively 20 cc. of plain infusion broth and 20 cc. of the same broth to which 1 per cent of glucose had been added, were sown with equal quantities of a young broth culture of the variant form of Strain Henson and were then placed in the incubator together. The hemolytic titer and the pH of samples from the two flasks were determined at intervals; and it will be seen from the two curves (I and II, Chart 2) that, in the culture which developed the larger quantity of acid owing to the fermentation of glucose, hemolysin appeared slowly, attained only a low titer and disappeared rapidly in comparison with the hemolysin of the less acid culture.

The next experiment shows that an acid reaction increases the rate at which hemolysin disappears from a filtrate.

A hemolytic filtrate (pH 7.4) was divided into two parts; the reaction of one part was adjusted to pH 8.0 by the addition of $\frac{N}{1}$ NaOH and the two portions were then placed in the incubator together. The hemolytic titers of the two portions of filtrate were determined before incubation, after 2½ hours incubation at 37°C. and again after a further 17½ hours at room temperature.

Table VI shows that the portion of filtrate which was adjusted to pH 8.0 retained its hemolysin longer than the untreated filtrate.

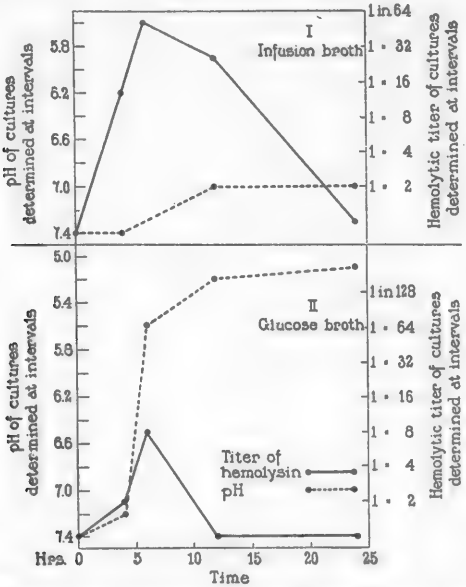

CHART 2. Showing the hemolytic titer and the pH of cultures of the same strain of hemolytic streptococcus. I. In infusion broth. II. In 1 per cent glucose broth.

Chart 3, which represents the result of a similar experiment, shows the rate of disappearance of hemolysin from two portions of a hemolytic filtrate which were kept in the ice box at 2°C. The portion of filtrate which was adjusted to pH 8.0 again retained its hemolysin longer than the untreated filtrate.

These experiments indicated that the rate of disappearance of hemolysin from cultures, and from filtrates, was dependent, to some

extent, on the reaction of the medium; and observations were therefore made to see whether the passage culture, which was non-hemolytic when exposed to air, produced more acid than the hemolytic glossy variant of the same strain. Shallow broth cultures of the two forms of Strain Henson which had been incubated long enough to produce the maximal quantity of hemolysin (6 to 8 hours) were filtered and the pH of the filtrates was determined: sometimes hemolytic filtrates from the glossy variant were more acid than hemolytic filtrates from the passage culture and, although slight differences of pH were usually found apparently due to the varying number of cocci which developed in the cultures, the reactions were always approximately equal and

TABLE VI.

Showing the Relation between the pH of a Filtrate and the Disappearance of Hemolysin.

Dilution of filtrate	Original hemolytic activity of filtrate		Hemolytic activity after 2½ hrs.		Hemolytic activity after 20 hrs.	
	pH = 7.4	pH = 8.0	pH = 7.4	pH = 8.0	pH = 7.4	pH = 8.0
1 in 2	++++	++++	++++	++++	+	+++
1 in 4	++++	++++	++++	++++	+	+++
1 in 8	++++	++++	+++	++++	+	++
1 in 16	++++	++++	+++	++++	−	++
1 in 32	++++	++++	++	+++	−	+
1 in 64	++++	++++	+	++	−	+
1 in 128	+++	+++	−	+	−	−

no constant preponderance of acid could be demonstrated in either culture.

The Cultivation of Different Forms of the Same Strain in Symbiosis.

The following experiments show the hemolytic titer of symbiotic cultures of the hemolytic and non-hemolytic forms of Strain Henson.

Three tubes of infusion broth, containing 20 per cent of normal horse serum, were sown in the following manner with young overnight cultures of the hemolytic and of the non-hemolytic forms of Strain Henson.

Tube 1 received 0.2 cc. of the non-hemolytic culture.

Tube 2 received 0.2 cc. of the hemolytic culture.

Tube 3 received 0.1 cc. of the non-hemolytic culture and 0.1 cc. of the hemolytic culture.

Ample oxygenation was assured by bubbling oxygen through the three cultures during the whole period of incubation which was continued for 5½ hours. The hemolytic titer of the three filtrates obtained from these cultures is shown in Table VII.

The pure culture of the hemolytic form contained hemolysin but the other two cultures contained no hemolysin.

The absence of hemolysin from the symbiotic culture might be explained by at least two hypotheses: (1) that the non-hemolytic form produced some substance in the presence of oxygen which not only destroyed its own hemolysin but was also capable of destroying

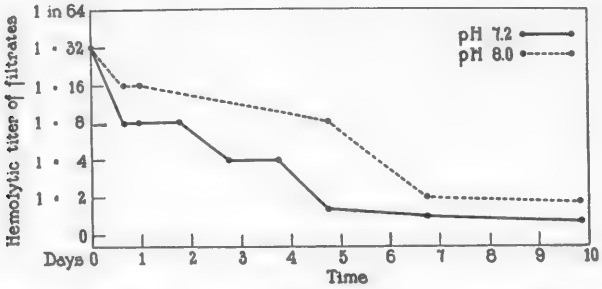

CHART 3. Showing the relation between the pH of a filtrate and the rate of disappearance of hemolysin.

the hemolysin of the glossy variant; (2) that the non-hemolytic cocci suppressed the multiplication of the hemolytic cocci.

To investigate the latter possibility various dilutions of the symbiotic culture were plated out on the surface of blood agar and incubated in the presence of oxygen; the number of hemolytic and of non-hemolytic colonies which developed on the plates showed that the two forms had multiplied at approximately the same rate, as 65 per cent of the 509 colonies counted were hemolytic. Differential counts of hemolytic and non-hemolytic colonies were facilitated by the characteristic translucence of the glossy hemolytic colonies and by the equally characteristic opacity of the non-hemolytic matt colonies. Table VIII gives the result of a similar experiment with the same strain of hemolytic streptococcus; but the conditions were modified by omitting the oxygenation of the cultures and by substituting cultivation in very shallow layers of broth. Three 2 liter Erlenmeyer flasks, each containing 10 cc. of serum-infusion broth, were sown with the same

volumes of hemolytic and of non-hemolytic cultures as in the previous experiment; and after 5½ hours incubation the cultures were centrifuged and the quantity of hemolysin in the supernatant fluids was determined.

It will be seen from Table VIII, first that the non-hemolytic culture formed some hemolysin in this experiment, possibly due to imperfect oxygenation, second that

TABLE VII.

Hemolysin in Filtrates Prepared from the Hemolytic and Non-Hemolytic Forms of Strain Henson and from a Symbiotic Culture of Both Forms.

Dilution of filtrates	Filtrates prepared from:		
	Pure culture of non-hemolytic form	Pure culture of hemolytic form	Symbiotic culture of both forms
1 in 2	−	++++	−
1 in 4	−	+++	−
1 in 8	−	++	−
1 in 16	−	+	−
1 in 32	−	−	−

TABLE VIII.

Hemolysin in Supernatant Fluid from Centrifuged Cultures of the Hemolytic and the Non-Hemolytic Forms of Strain Henson and from a Symbiotic Culture of Both Forms.

Dilution of supernatant fluid	Supernatant fluid from:		
	Pure culture of non-hemolytic form	Pure culture of hemolytic form	Symbiotic culture of both forms
1 in 2	++	++++	++++
1 in 4	+	++++	++
1 in 8	−	++++	+
1 in 16	−	++++	−
1 in 32	−	+++	−
1 in 64	−	++	−
1 in 128	−	+	−
1 in 256	−	−	−

the glossy variant was actively hemolytic, and, third that the symbiotic culture contained little more hemolysin than the pure culture of the non-hemolytic form. Differential counts showed that the symbiotic culture contained more than 50 per cent of hemolytic cocci.

The next experiment was done to determine the action of the non-hemolytic form of Strain Henson in symbiotic culture with an unrelated strain of hemolytic streptococcus (S43).

Two shallow layers of serum-infusion broth were sown with 0.2 cc. and 0.1 cc. of a culture of Strain S43 respectively; to the second was also added 0.1 cc. of a culture of the non-hemolytic form of Strain Henson. After 6 hours incubation the cultures were filtered and titrated for hemolysin content, as shown in Table IX.

Differential counts showed that the symbiotic culture contained 45 per cent of hemolytic cocci; a proportion which was not represented by the hemolytic titer

TABLE IX.

Hemolysin in Filtrate Prepared from an Unrelated Strain of Hemolytic Streptococcus (S43) and in Filtrate Prepared from the Same Strain in Symbiosis with the Non-Hemolytic Form of Strain Henson.

Dilution of filtrate	Pure culture of hemolytic streptococcus Strain S43	Symbiotic culture of hemolytic streptococcus Strain S43 and the non-hemolytic form of Strain Henson
1 in 2	++++	+++
1 in 4	++++	+++
1 in 8	++++	++
1 in 16	++++	+
1 in 32	+++	−
1 in 64	+++	−
1 in 128	+++	−
1 in 256	++	−
1 in 512	+	−
1 in 1,024	−	−

of the filtrate as the pure culture of Strain S43 contained considerably more than twice the quantity of hemolysin found in the symbiotic culture.

It was difficult to compute the quantity of hemolysin which might be expected to develop in a symbiotic culture if the non-hemolytic fraction of the mixed culture was inactive; and in order to gain some information on this point, and at the same time to reinforce the evidence already obtained that the destruction of hemolysin was not due to oxidation by peroxide, symbiotic cultures, composed of mixtures *of Streptococcus hæmolyticus* and *Streptococcus viridans*, were prepared. Five 250 cc. Erlenmeyer flasks each containing 10 cc. of serum-infusion broth were sown with the following quantities of culture:

Flask 1 received 0.2 cc. of the hemolytic glossy variant of Strain Henson.

Flask 2 received 0.2 cc. of the non-hemolytic form of Strain Henson.

Flask 3 received 0.2 cc. of *Streptococcus viridans* Strain A179.

Flask 4 received 0.1 cc. of the hemolytic glossy variant of Strain Henson and 0.1 cc. of the non-hemolytic form of Strain Henson.

Flask 5 received 0.1 cc of the hemolytic glossy variant of Strain Henson and 0.1 cc. of *Streptococcus viridans* Strain A179.

After 5½ hours incubation the cultures were tested for peroxide and then filtered immediately; Table X gives the quantities of hemolysin which the five filtrates contained. All the cultures gave negative peroxide tests with the single exception of the pure culture of *Streptococcus viridans* which gave a faintly positive potato-benzidine reaction.

The hemolytic form of Strain Henson produced, as usual, a good hemolytic filtrate; the culture of *Streptococcus viridans*, and also the culture of the non-hemolytic form of Strain Henson, produced no hemolysin. The hemolytic titer

TABLE X.

Hemolysin in Filtrates Prepared from Pure Cultures and from Symbiotic Cultures of Strain Henson and Streptococcus viridans.

Dilution of filtrates	Hemolytic titer in filtrates prepared from:				
	Pure cultures of:			Symbiotic cultures of hemolytic form of Strain Henson and	
	Hemolytic form of Strain Henson	Non-hemolytic form of Strain Henson	*Streptococcus viridans*	Non-hemolytic form of Strain Henson	*Streptococcus viridans*
1 in 2	++++	−	−	+++	++++
1 in 4	++++	−	−	++	++++
1 in 8	++++	−	−	+	++++
1 in 16	++++	−	−	+	++++
1 in 32	++++	−	−	−	++++
1 in 64	+++	−	−	−	+++
1 in 128	+++	−	−	−	+++

of the symbiotic cultures showed that the hemolysin of the glossy variant was partially destroyed in symbiosis with the non-hemolytic form of Strain Henson; but symbiosis with *Streptococcus viridans* did not affect its output of hemolysin.

Differential counts on both the symbiotic cultures showed that the rate of multiplication of the hemolytic cocci and of the non-hemolytic cocci was approximately the same in each case.

These experiments tend to show that the non-hemolytic form of Strain Henson when cultivated in symbiosis with hemolytic cocci not only destroys its own hemolysin in the presence of oxygen, but also destroys the hemolysin of the associated hemolytic cocci.

Unfortunately more conclusive evidence of the destruction of hemolysin by the non-hemolytic form of Strain Henson was not obtained. Filtrates, prepared from the hemolytic form and from the non-hemolytic form, were mixed in equal proportions and a control was prepared by mixing together the same volumes of hemolytic filtrate and broth. Periodical titrations of these two mixtures showed that, when the pH was adjusted to the same level, hemolysin disappeared from the two mixtures at approximately the same speed.

Cultivation of the non-hemolytic forms in hemolytic filtrates did not give any further information, as the alteration of pH by the metabolism of the cocci was sufficient to cause hemolysin to disappear from the culture more rapidly than from the sterile control filtrate.

It is therefore uncertain whether the non-hemolytic form destroys the hemolysin formed by hemolytic streptococci or whether it produces conditions in the medium which prevent the formation of hemolysin.

DISCUSSION.

A number of workers have reported the mutation of *Streptococcus hæmolyticus* to *Streptococcus viridans* by animal passage. A similar change is recorded in this paper; but in this instance the loss of hemolytic activity was conditional on ample oxygenation and the organisms retained their hemolytic power in the absence of oxygen. It is probable that some of the previously reported "mutants" were also modified forms of hemolytic streptococci which, although they formed green colonies on blood agar, were possibly distinguishable from typical strains of *Streptococcus viridans* by their hemolytic activity in anaerobic culture.

The hemolytic streptococci, modified by animal passage, produced less methemoglobin on blood agar than typical green streptococci; and it would have been difficult to decide, on this criterion alone, whether they should be classified as green streptococci or as indifferent streptococci. The passage culture reverted to the hemolytic form after prolonged subcultivation on artificial media; and this reversion, coupled with the fact that the culture was always hemolytic in the absence of oxygen, served to identify the organism as a modified form of *Streptococcus hæmolyticus*.

The passage culture produced more peroxide than either of the

hemolytic forms of the same strain; and an attempt was therefore made to establish a causal relationship between the accumulation of peroxide and the disappearance of hemolysin. Periodical observations on broth cultures of the three forms of this strain showed that disappearance of hemolysin was concomitant with accumulation of peroxide; but attempts to demonstrate the destruction of hemolysin by peroxide were unsuccessful. It may be suggested that continued production of peroxide over a relatively long period, as by bacterial metabolism in a culture, is necessary for the destruction of hemolysin and that this condition was not established in the experiments recorded; but this explanation is untenable when we consider that anaerobic cultures of hemolytic streptococci, though unable to form peroxide, lost their hemolysin at the same rate as aerobic cultures which produced peroxide.

It is interesting to note that peroxide is preserved in an acid reaction while hemolysin is destroyed under the same condition; and it might be supposed that the pH of a culture determines the relationship between disappearance of hemolysin and appearance of peroxide, but this explanation seems inadequate because the different forms of hemolytic streptococci were approximately equal in their rate of acid production.

We are therefore forced to the conclusions that the hemolysin of *Streptococcus hæmolyticus*, in contrast to the apparently similar hemotoxins of pneumococci and other bacteria, is not destroyed by oxidation; and that the concomitant disappearance of hemolysin and appearance of peroxide have no causal relationship, though they may both be dependent on some unknown common cause.

The passage culture, though hemolytic in the absence of oxygen, establishes some unknown condition in the presence of oxygen which abolishes hemolysin and, when grown symbiotically with an ordinary strain of hemolytic streptococcus which is capable of forming hemolysin in the presence of oxygen, it reduces the hemolytic titer of the symbiotic culture either by destroying hemolysin or by preventing its formation.

SUMMARY.

From one strain of hemolytic streptococcus three forms were isolated, which produced three different degrees of hemolysis on the surface of blood agar in the presence of oxygen.

The original form was moderately hemolytic; the glossy variant was more hemolytic than the original form; and the third form, obtained by passing the original culture through mice, was non-hemolytic.

Under anaerobic conditions all three forms were hemolytic.

The non-hemolytic passage culture, in the presence of an ample supply of oxygen, not only destroyed its own hemolysin, which only appeared under anaerobic conditions, but was also able to destroy the hemolysin of other cultures of hemolytic streptococci.

It is possible that these observations may throw some light on experiments reported by a number of workers showing that *Streptococcus hæmolyticus* can be transmuted to *Streptococcus viridans* by animal passage.

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A STUDY OF THE MECHANISM OF RECOVERY FROM LOBAR PNEUMONIA.

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Investigations into the mechanism of recovery from lobar pneumonia of recent years have brought forward an increasing amount of data to show that the principal evidence of acquired immunity consists in the development of humoral immune substances at or about the time of crisis. Yet the majority of authors writing on this subject express considerable caution in drawing conclusions as to the causal relation between the immune bodies and the recovery process even though certain of these substances or reactions have been shown to be directly concerned in the destruction of pneumococci and protection of the body from invasion. The reasons for this reservation of judgment are principally as follows: First, the occurrence of immune substances in the serum has not been observed as a constant phenomenon in all cases recovering from pneumonia; second, the appearance of these immune bodies, while generally coinciding with the onset of recovery, may occur 1 or 2 days before crisis or in some cases not until afterwards; third, serum immunity has been detected infrequently in cases terminating fatally; fourth, while the promotion of phagocytosis and intracellular destruction has been found to be one of the chief manifestations of acquired antipneumococcus serum action, the evidence for pneumococcidal power of the blood at the time of recovery is not generally considered as convincing. Furthermore, doubt has been cast on the importance of phagocytosis as a factor in the disposal of pneumococci in the body by the failure to demonstrate any degree of engulfment of these microorganisms by the cells of the lung exudate secured from patients during the period of recovery.

Finally, the fact that the introduction of large quantities of immune serum containing a high concentration of specific antibodies has been found by a number of investigators to be ineffective in cases of pneumonia after the 2nd or 3rd day of the disease, raises the question as to the ultimate rôle played by these serum substances.

The above considerations have led certain workers to seek other explanations for the sudden termination of infection in lobar pneumonia. Changes in the H ion concentration of pneumonic exudate in relation to the acid death-point of pneumococci have been studied (1). Investigation of the mobilization of enzymes in the blood at the time of crisis (2) have also brought to light interesting facts but they do not afford a satisfactory interpretation of the recovery process.

Our findings in a study of experimental pneumococcus infection in cats and rabbits (3) suggested that it would be profitable to pursue further by the same methods the inquiry as to the relation of acquired humoral immunity to the mechanism of recovery from lobar pneumonia in man. It was found that at the time of recovery from experimental disease there appeared constantly in the serum, immune properties characterized not only by opsonic, agglutinative, and mouse-protective activity, but also by the power to promote to a marked degree the killing of virulent pneumococci in rabbit serum-leucocyte mixtures. None of these changes occurred in animals with fatal infection. The degree of pneumococcus-killing power potential in the recovered animal's blood made it appear that this action played an important part in the disposal of pneumococci. While the development of humoral immune bodies was usually more marked and could be more closely related to the onset of recovery in the pneumococcus-resistant animal (the cat) than in the susceptible animal (the rabbit), yet the latter showed the same type of serum immune change and hence it seemed probable that in the human, whose resistance lies somewhere between that of these two animals, a similar immune reaction occurs.

Methods.

The methods employed in carrying out the pneumococcidal, opsonic, and agglutinative tests have been described in previous publications (4–6). The pneumococcidal-promoting power of the serum was tested by adding it in progressive dilution to rabbit serum-leucocyte mixtures containing small quantities of

TABLE I.

Determination of Pneumococcidal-Promoting Power of Serum during Course of Lobar Pneumonia, Pneumococcus Type II.

Human serum dilution 0.1 cc. + normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Serum sample in relation to lysis	Amount of standard pneumococcus suspension	Dilutions human serum	Growth as shown by color changes at hrs.*			Pneumococci in stained film at 72 hrs.
			16	48	72	
2 days before	10^{-6}**	1–10	++++			+
	"	1–20	++++			+
1 day before	"	1–10	++++			+
	"	1–20	++++			+
Day beginning	"	1–10	0	0	0	0
	"	1–20	0	++++		+
	"	1–40	++++			+
	"	1–80	++++			+
	"	1–160	++++			+
Day completed	"	1–40	0	0	0	0
	"	1–80	0	0	0	0
	"	1–160	0	0	0	0
	"	1–320	0	0	0	0
	"	1–640	++++			+
	"	1–1280	++++			+
3 days after	"	1–40	0	0	0	0
	"	1–80	0	0	0	0
	"	1–160	0	0	0	0
	"	1–320	0	0	0	0
	"	1–640	++++			+
	"	1–1280	++++			+
12 days after	"	1–20	0	0	0	0
	"	1–40	++++			+
	"	1–80	++++			+
	"	1–160	++++			+
	"	1–320	++++			+
	"	1–640	++++			+
Controls	10^{-7}	0	++++			+
	"	0	++++			+

* Degrees of methemoglobin formation.

** This amount represents 500 to 1000 pairs of pneumococci.

highly virulent pneumococci. Since these mixtures have no growth-inhibitory action on such organisms, any pneumococcidal effect which occurs may be attributed directly to the added test serum. The amounts of the various constituents used in the reaction are given in Table I. Opsonic and agglutinative tests were performed by mixing pneumococci which had completed their active growth phase[1] with serum, in a ratio of 1 part of pneumococcus suspension to 20 parts of serum. After sensitization for 1 hour the pneumococci were then separated from the serum by centrifugation, the sedimented mass broken up with a capillary pipette, macroscopic agglutination noted, and phagocytic tests performed in the usual way with especially prepared rabbit leucocytes. The degrees of opsonic action and agglutination were noted in terms of plus signs ranging from + slight but definite, to ++++ which was very marked. Since all the specimens of serum from one patient, inactivated after separation and kept on ice, were tested at one time it was possible to make comparative readings on them. The different tests, pneumococcidal, opsonic, agglutination, and mouse protection, were usually made on the same day. Occasionally this was not possible.

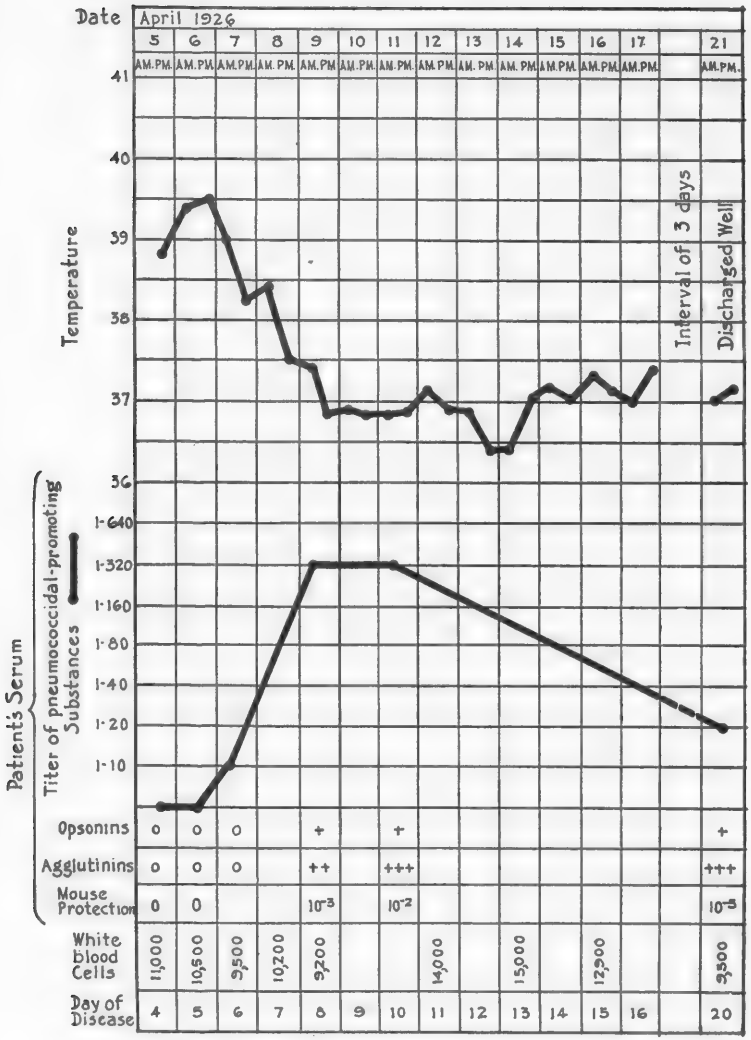
The pneumococci used in the tests with Type I sera were highly virulent strains. The homologous organisms isolated from both blood and sputum as well as a stock culture were used. In the tests with serum from pneumonia Type II and Group IV it was necessary to use the serum and leucocytes of young rabbits as none of the strains from these patients were virulent, nor could they be made virulent for full grown rabbits (7). Controls with young rabbit serum and leucocytes showed no opsonic or agglutinative action for these Type II and Group IV strains of pneumococci which were highly virulent for the young animal.

Clinical Cases.

The following study represents observations made on seventeen cases of lobar pneumonia comprising five cases of Pneumococcus Type I, six of Type II, two of Type II atypical, and four Group IV. Sixteen of the patients recovered. One died.

It may be stated briefly at the outset that the results of the study in human cases were essentially the same as those obtained in cats with experimental pneumococcus infection. Furthermore no constant differences in reaction were observed between the three types of pneumonia studied. The blood serum in the early stages of the disease was found to be without pneumococcus immune principles. Then, about the time of crisis, the serum acquired demonstrable

[1] Type II pneumococci, however, were used in the active growth phase. The significance of the growth phase in relation to opsonic and agglutination tests is discussed in an earlier publication (*J. Exp. Med.*, 1927, xlvi, 239).



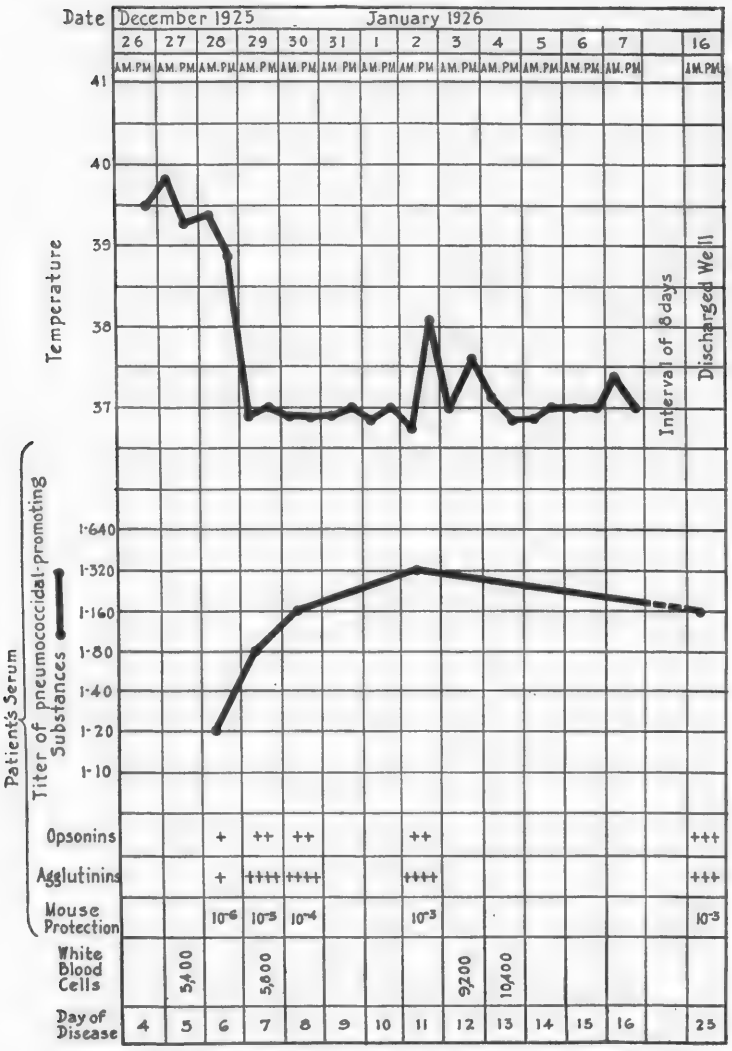
TEXT-FIG. 1. Case 13455. Lobar pneumonia, Pneumococcus Type II.

pneumococcidal-promoting power as well as opsonic, agglutinative, and mouse-protective properties. Text-fig. 1 illustrates well the sequence of changes found to occur in cases where the observations were begun early in the disease course. Tests on the blood specimens taken from this patient on the 4th and 5th days of disease were negative (Table I). On the 6th day, which marked the beginning of the fall in temperature, the serum showed the titer of pneumococcidal-promoting substances of 1:10. Opsonins and agglutinins were not demonstrable at this time. On the 8th day when the temperature had reached normal the titer of pneumococcidal-promoting substances had risen to 1:320 and opsonins, agglutinins, and mouse-protective bodies were evident.

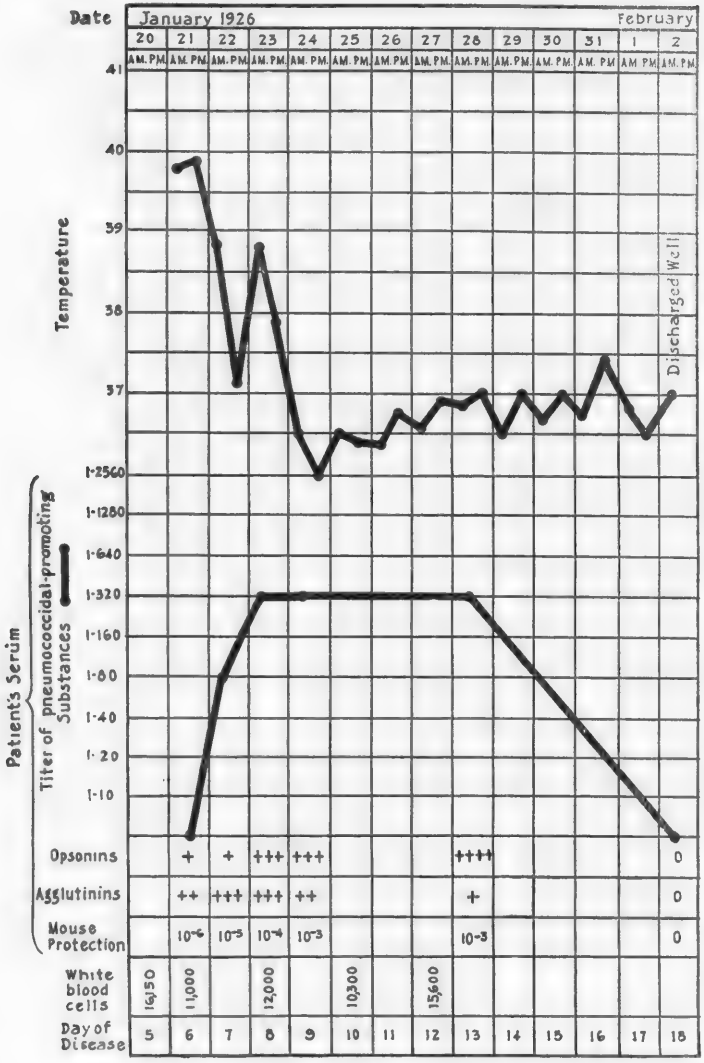
The occurrence of demonstrable immune substances in the blood as shown especially by the pneumococcidal-promoting power, appeared to coincide generally with the beginning of defervescence. In only one instance (Text-fig. 4) were immune properties found in the serum more than a very short time (hours) before the temperature began to fall. In one other case pneumococcidal-promoting substances failed to appear until after the temperature had reached normal. It is quite possible that a more complete series of observations made immediately preceding the initiation of recovery might show a constantly earlier appearance of serum immune properties, although the results of tests such as are exhibited in Text-figs. 1 and 3, in which the immune reactions were found to be present initially to only a slight degree, suggest that they had not been detectable much earlier. In support of this supposition is the fact that once this immune property appeared in the blood it showed in most instances a rapid quantitative increase.[2]

The titer of pneumococcidal-promoting substances usually reached its height within 48 to 72 hours. Then after some days at this high level it began to fall. Just how long the serum continued to show immune properties was not determined but that the persistence of passive immunity varies greatly from patient to patient was shown by the finding in some cases that all traces of humoral immunity had

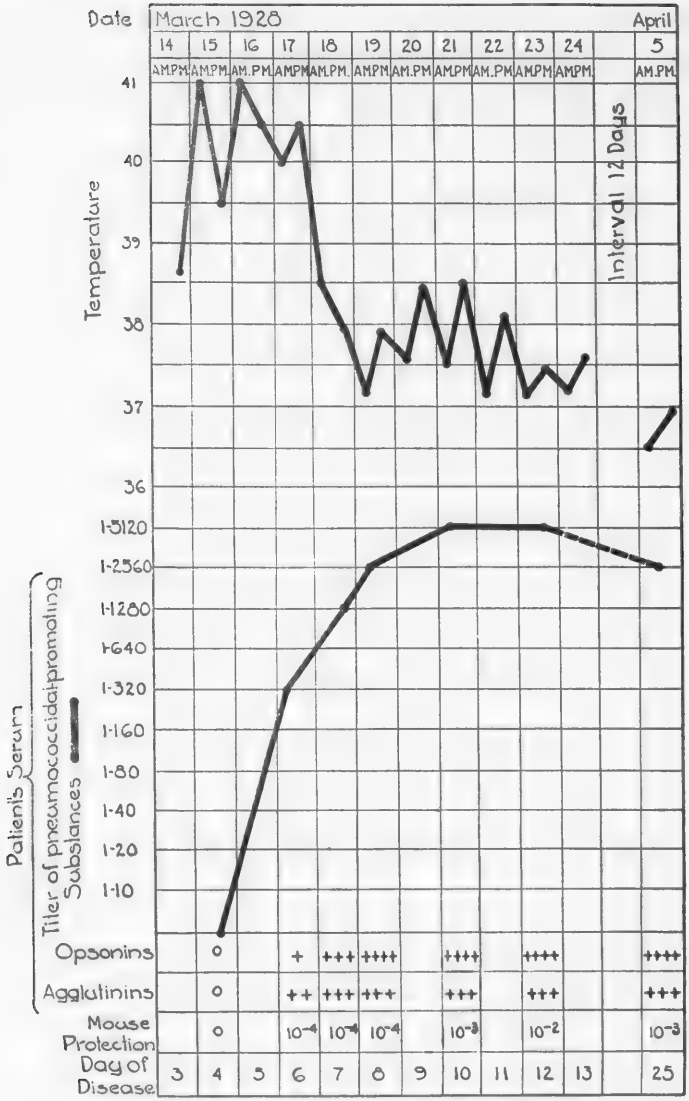
[2] Our observations in experimental animals (3) in which a rapidly diminishing blood invasion occurred 24 to 48 hours before the termination of the disease make it seem highly probable that immune bodies are elaborated and perhaps present in low concentration for some time before crisis.



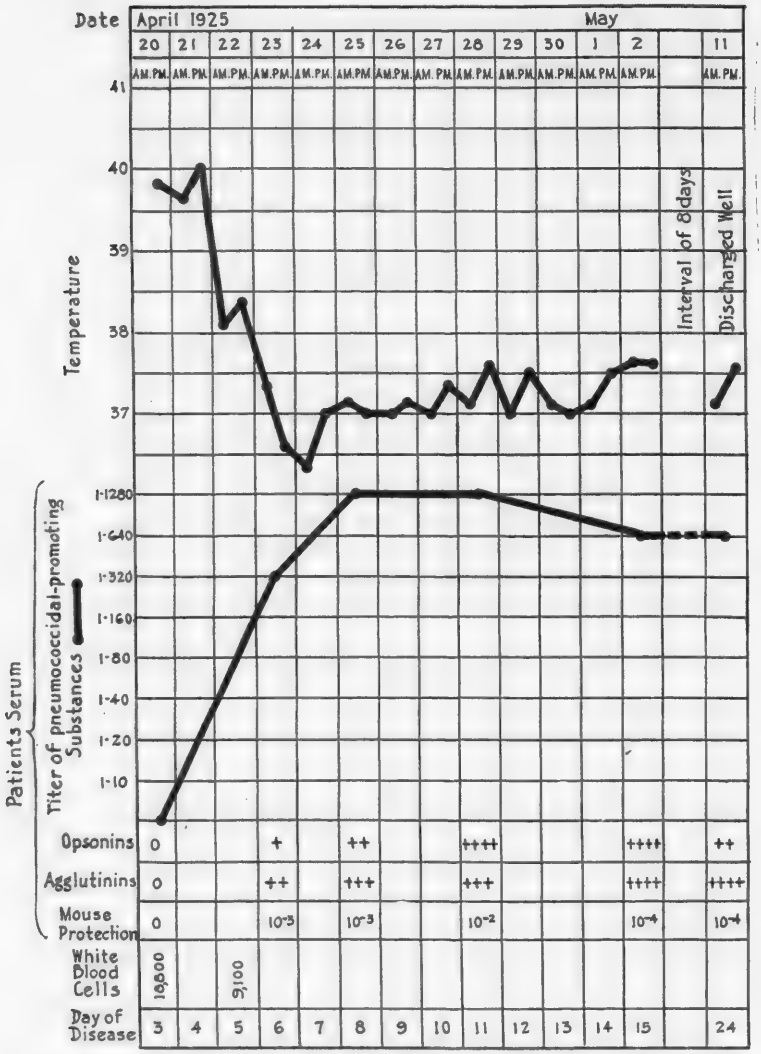
TEXT-FIG. 2. Case 12656. Lobar pneumonia, Pneumococcus Type II.



TEXT-FIG. 3. Case 12827. Lobar pneumonia, Pneumococcus Type I.



TEXT-FIG. 4. Case 3193. Lobar pneumonia, Pneumococcus Type II atypical.

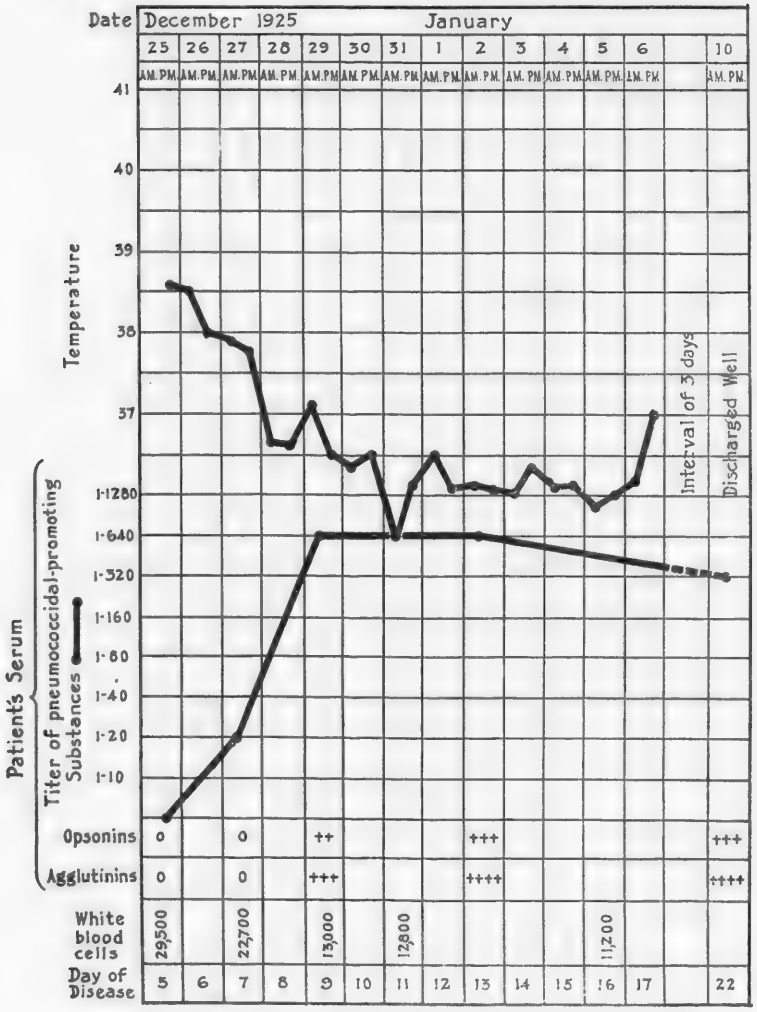


TEXT-FIG. 5. Case 10608. Lobar pneumonia, Pneumococcus Type II.

disappeared within 10 days after crisis (Case 12827, Text-fig. 3), while in others, and these constituted the majority, at the end of weeks or months serum immune properties were still evident though much diminished. Note Case 4960, Text-fig. 7, tested after 74 days. The persistence of immune substances in the serum seemed to be associated to a certain degree with the initial intensity of immune body production. Thus, those patients in whom serum immunity was demonstrable for the longest periods showed a relatively high pneumococcidal-promoting serum titer at outset, and those in whom the serum immunity disappeared earlier showed an initial low or at most moderately elevated titer. However, these observations are too few to permit any conclusions concerning this point.

Only one patient of the sixteen recovering showed a positive blood culture. This was taken on the day before the appearance of serum immune substances which occurred at the time of crisis, hence it was not possible in this series of cases to determine whether the occurrence of detectable pneumococcidal-promoting substances in the blood marked the termination of blood invasion as was always found to be the case in cats recovering from experimental pneumococcus infection.

In the majority of the cases all the different manifestations of serum immunity became demonstrable at the same time as in Cases 12656 and 3193, Text-figs. 2 and 4. In others, the appearance of pneumococcidal-promoting power in the serum seemed to precede or occasionally lag behind the occurrence of opsonic, agglutinative, and mouse-protective action (Text-figs. 1, 3, and 6). These variations may, however, be more apparent than real since the different means employed to bring out the several reactions may not be strictly comparable in their power to detect minute traces of the immune substance or substances. Furthermore, a comparison of the changing intensity of the four reactions through the period of observation revealed a general parallelism. This was closest between the pneumococcidal-promoting power and the mouse-protective action which are more susceptible to accurate quantitative estimation than are the opsonic and agglutinative reactions as carried out in this work. It was found frequently, especially at the beginning of recovery, that equal parts of serum and pneumococcus suspension failed to bring out opsonic activity while employing 20 parts of serum to 1 of suspension produced a well defined reaction.



TEXT-FIG. 6. Case 7797. Lobar pneumonia, Pneumococcus Type II.

In striking contrast to the findings in patients recovering, are those made on the case progressing to a fatal termination (Text-fig. 8). Repeated tests of the blood serum made during the course of the disease in this patient failed to reveal any evidence of the development of immune properties. Tests for opsonic, agglutinative, and mouse-protective action were not made in this case but judging from the findings in experimental pneumococcus infection (3), it is probable that these reactions were also lacking here. There was a persistent slight blood invasion but it did not increase during the course of the disease.

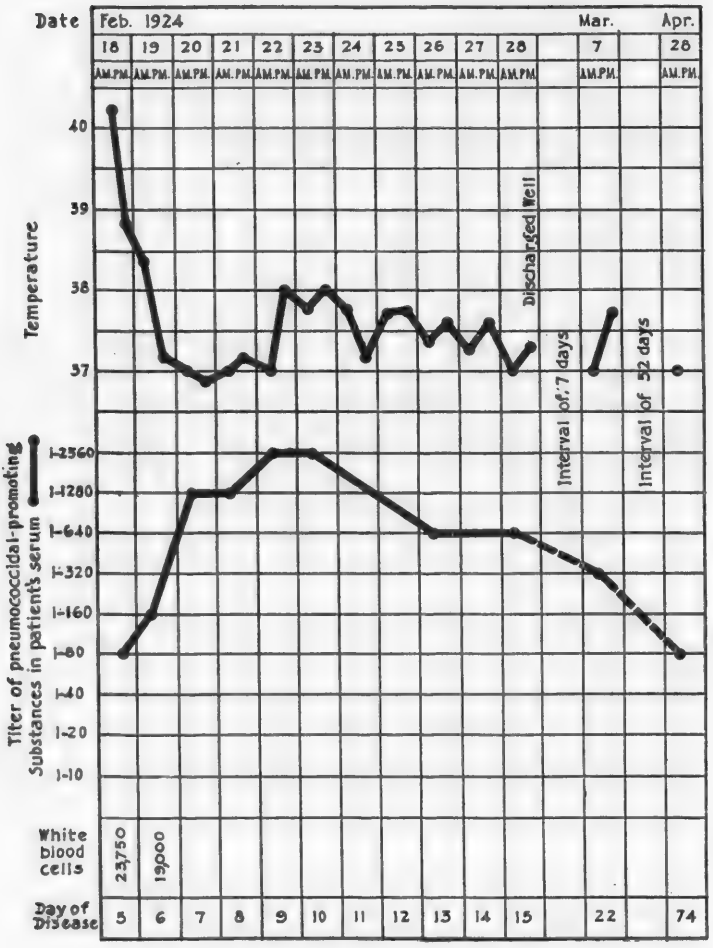
Specificity of Immune Substances.

The pneumococcidal-promoting substances occurring in the serum at recovery were found to be strictly type-specific. Pneumococcidal tests in which one serum was used with several types of pneumococci and also several types of serum with one type of pneumococcus, showed killing of the pneumococci only in those tubes containing the homologous serum and organisms. The opsonic and agglutinative reactions were also found to be specific to type.

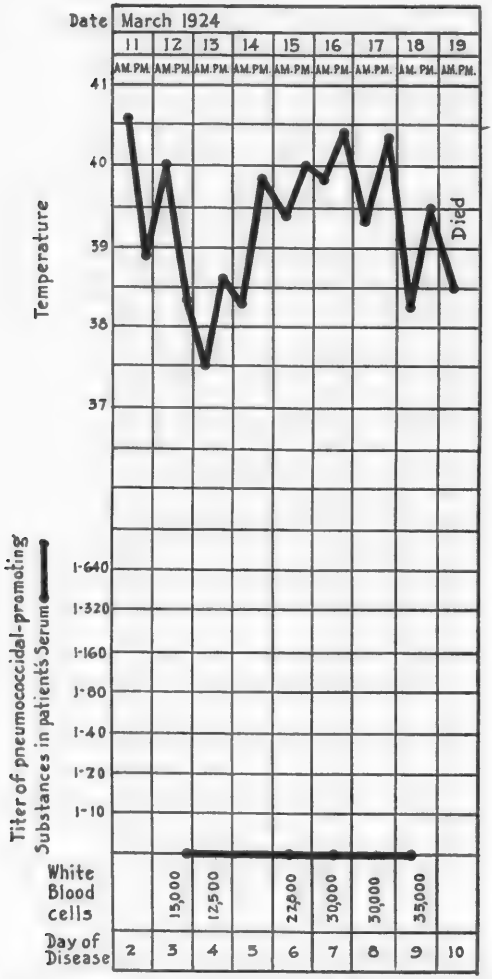
White Blood Cells.

The observations on the leucocytic response in this series of pneumonia patients are much less complete than those made in the experimental infected animals described in the preceding paper to which reference has been made (3). However, certain points are apparent and these are in general similar to the findings in the experimental disease. In the majority of the human cases there was a moderately increased or high white count just before the onset of recovery. In others, however, the number of leucocytes remained about normal and in one instance slightly below normal during the critical period (see Text-figs. 1 and 2). The fatal case showed an increasing white count towards the end of the disease reaching 35,000 the day before death.[3] These findings are in agreement with the inferences drawn from the animal studies, namely, that the leucocytes probably play a secondary rôle in the mechanism of recovery from pneumococcus infection, but

[3] There was no evidence of complications in this patient although this could not be definitely determined since an autopsy was not obtained.



TEXT-FIG. 7. Case 4960. Lobar pneumonia, Pneumococcus Type I.



TEXT-FIG. 8. Case 4983. Lobar pneumonia, Pneumococcus Type II.

the well substantiated clinical observation that a low white count is generally to be considered as of unfavorable prognostic import, indicates that a certain minimum number of white blood cells are necessary to provide suitable conditions for recovery.

Complications and the Presence of Immune Substances.

Two of the patients in this series, a Group IV and a Type II pneumococcus pneumonia, developed empyema at a time when there were demonstrable pneumococcidal-promoting properties in the serum. In one instance the titer was low, 1:40, in the other it was 1:640. Both patients recovered.

DISCUSSION.

While the above findings do not warrant the conclusion that the development of specific antipneumococcus properties occurs constantly in the serum of all patients recovering from lobar pneumonia, they do indicate that these serum changes are of very common occurrence and perhaps are present to varying degrees in every case. The fact that certain workers, Chickering (8), Clough (9), Lister (10), and others, have failed to demonstrate the presence of opsonins and agglutinins in a small per cent of pneumonia cases at the time of crisis may simply indicate the occurrence of considerable variation in the degree of antibody response. We have shown that the use of equal parts of serum and pneumococcus suspension employed in the earlier studies may fail to bring out a low concentration of opsonins and agglutinins. Chickering mentions that in some of his cases agglutinins were detected on 1 day only. However, the absence of mouse-protective action in the critical and postcritical serum observed by certain investigators in occasional cases recovering from lobar pneumonia is of greater significance on account of the marked sensitiveness of this test.[4] Dochez (11) found in a series of ten recovering cases that the serum of one failed to show protective power against the homologous organism. Clough (12) studying twelve cases found protective action in the serum of only nine. In the three patients in whom the serum showed no protection, only one serum specimen was obtained in each case and this, 1 to 3 days following recovery. Other authors, and most recently

[4] Mouse protection seems to be fully as delicate an indication of the presence of antipneumococcus immune substances as is the test for pneumococcidal action.

Baldwin and Rhoades (13), have found protective substances constantly in the serum of recovering patients.

Previous studies by other investigators have shown that in the majority of cases the appearance of circulating immune bodies coincides closely with the time of crisis or lysis. But in a small percentage of pneumonia patients immune substances have been detected 1 to 2 days beforehand. Baldwin and Rhoades (13) found protective bodies in twelve out of twenty cases at least 2 days prior to the day of crisis. However, a number of their reported cases had received specific therapy of one kind or another so that it is not clear how many of them developed the passive immune properties spontaneously. Does this occasional finding of immune properties in the serum sometime before crisis constitute a serious objection to the assumption that immune body development is related intimately to the mechanism of recovery? While the relationship between demonstrable humoral immunity and recovery would be much clearer if serum immune substances always appeared at or immediately before crisis, the fact that they may be present in the blood a day or two before the apparent beginning termination of the disease process does not seem to provide a sufficient reason for denying them an important rôle in the recovery mechanism. We know relatively little about conditions in the body which influence the functioning of opsonins, protective bodies, etc. Furthermore, structural peculiarities in the local lesion may well produce variation in the rate at which the immune substances penetrate or there may be other factors of a more general nature upon which the effective action of these antipneumococcus bodies largely depends. May not the early appearance of acquired immune bodies in a given case of lobar pneumonia represent a condition somewhat analogous to that which is frequently observed after the introduction of antipneumococcus serum Type I? Following the injection of an adequate amount of immune serum the blood acquires immune properties but the crisis may not occur for several days. However, the blood invasion ceases and the pneumonic process stops spreading. It has been shown by Baldwin and Cecil (14) that the appearance of protective bodies in the blood of spontaneously recovering cases marks the termination of blood invasion, a finding with which our observations in experimental pneumococcus infection agree. It is not possible that the spread of the pneumonic process

also ceases at this time? Our one patient in whom humoral immunity was detected 12 to 18 hours before crisis (Text-fig. 4) did show physical signs of beginning resolution 24 hours before the critical fall in temperature. We have found no observations on this point in the literature.

There exists also the possibility that the termination of the disease depends on the development of antitoxic as well as antibacterial immunity, as was suggested years ago by Cole (15), Wadsworth (16), and others.

Failure to detect immune substances until 24 hours or more after crisis has been reported less frequently than their early appearance. This condition might be due to an unusually marked neutralization of perhaps a rather low concentration of these bodies at the time of recovery.

The finding of antipneumococcus immune properties in the serum of patients going on to a fatal termination has been confined as far as we can determine to cases developing complications. In Baldwin and Cecil's (14) three fatal cases showing circulating humoral immune substances there was a complicating empyema. In Clough's (9) one reported case, an active endocarditis was probably present. The development of such secondary foci of infection in patients in whom the pneumonic process has been checked and who show evidence of considerable serum immunity is a further indication of the complexity of the recovery mechanism.

Of even more importance than the finding of antipneumococcus substances in the serum during recovery is information as to their mode of action. The demonstration in this study that a serum showing such properties possesses the power to promote the destruction of highly virulent pneumococci provides more direct evidence of the function of these immune bodies than has heretofore been obtained. That immune serum promotes phagocytosis and intracellular digestion in the body as it does in the test-tube seems highly probable although it should be pointed out that we have made no determinations of the activity of the pneumonic leucocytes. Investigations in this field by other workers (17–19) have shown that the leucocytes apparently function actively during the height of the disease.[5]

[5] A discussion of this phase of the subject is taken up in an earlier publication (3).

The fact that the above findings in human cases of lobar pneumonia are in practically entire accord with the observations made in cats undergoing experimental pneumococcus infection enhances considerably the significance of the development of serum immune bodies at the time of crisis. In the cat it was possible to show that the acquisition of humoral immunity was associated with greatly increased antipneumococcus resistance. Furthermore variations in the degree of acquired immunity were found to be associated in a general way with the corresponding fluctuations in concentration of demonstrable serum immune bodies. But while these observations suggest that the reaction of the body to pneumococcus invasion is the same in animals and in man despite the great dissimilarity in the character of the lesions produced, they do not indicate certainly that the mechanism of recovery is identical in both instances. The peculiar character of the local lesion in lobar pneumonia would seem to introduce certain special conditions to be reckoned with in the human body's defense against this organism and which may involve the operation of processes other than those essential for recovery in less intensely localized pneumococcus infection. In a discussion of this question, Cole (20) suggests that the destruction of pneumococci in the lung lesion may depend on local factors quite different from those responsible for the destruction of the bacteria in the circulating blood. He mentions the marked changes which occur in the resolving lung exudate, the solution of fibrin acting as a possible relief from tension and affording an outlet for the exudate and the production of chemical compounds, soaps, fatty acids, etc., shown to have a destructive action on pneumococci. However, he points out the difficulty in inferring that the crisis depends mainly on the resolution of the exudate, in that these two processes do not necessarily occur synchronously. Cole's conclusion (21) that recovery depends largely on an adequate concentration of immune bodies in the blood is supported by the observations presented in this paper.

SUMMARY.

Employing a method devised for the investigation of natural immunity and experimental pneumococcus infection, a study has been made of the serum immune changes occurring during the course of

lobar pneumonia due to Pneumococcus Types I and II and Group IV, in man. It was found that at the time of crisis or lysis the blood serum acquired constantly the property of promoting pneumococcus killing to a relatively marked degree. Other evidences of antipneumococcus reaction—mouse protection, opsonins, and agglutinins—were also demonstrable in the blood at this time. These immune changes appeared in the majority of cases at the beginning of recovery and failed to occur when the disease terminated fatally. The fact that these observations in human cases are practically identical with previous findings in the experimental disease in cats, enhances considerably the significance of the development of serum immune bodies at the time of crisis since in the experimental animal it was possible to show that the acquisition of passive immunity was associated with greatly increased antipneumococcus resistance.

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14. Baldwin, H. S., and Cecil, R. L., *J. Am. Med. Assn.*, 1926, lxxxvii, 1709.
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16. Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 78.
17. Tunnicliff, R., *J. Infect. Dis.*, 1911, viii, 302.
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NITROGEN METABOLISM OF NORMAL AND SARCOMATOUS[1] FIBROBLASTS IN PURE CULTURES.

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It has been reported that the proteoses of Witte's peptone and the primary split products of many different proteins, although not a complete nutritive medium, cause a rapid proliferation of the fibroblasts migrating from fresh embryo heart.[2,3] Experiments on the fractionation of embryo juice, which promotes, as is well known, the unlimited growth of certain tissues *in vitro*, demonstrated that fibroblasts utilize the protein fraction.[4] On the other hand, they proliferate for only a short time when fed upon the amino acids present in the embryo juice,[5] or formed by hydrolysis of the proteins. Therefore, the hypothesis was proposed that enzymes present in the embryo juice, or within the cells, hydrolyze the proteins, and that the proteoses so formed determine the growth of the tissues. In the experiments described in the present paper, an attempt was made to ascertain whether some one fraction of the proteoses, or the proteoses as a whole, are responsible for growth and also whether other fragments of the protein molecule, such as peptones, peptides, and amino acids, contribute to cell nutrition.

[1] The sarcomatous fibroblasts referred to in this paper are from a pure strain of malignant fibroblasts from rat Sarcoma 10 of the Crocker Foundation, which have been cultivated *in vitro* for 18 months.

[2] Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503.

[3] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 353.

[4] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 387.

[5] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 397.

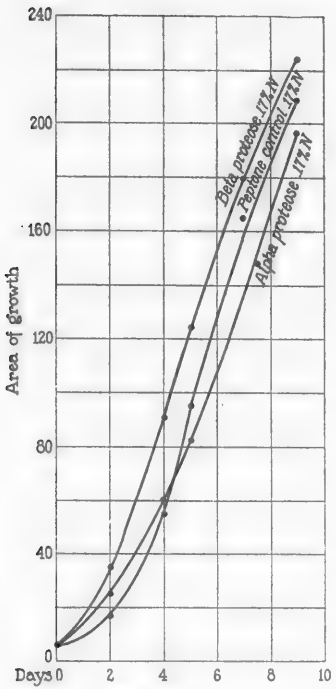
Action of the Proteose Fractions on Normal and Sarcomatous[6] Fibroblasts.

Witte's peptone was separated into heteroproteose by dialysis, and alpha and beta proteoses[7] by alcoholic precipitation. The alcohol was removed by evaporation *in vacuo*, and the fractions were dissolved in Tyrode solution, or rendered isotonic by the addition of the salts of Ringer solution and tested at pH 7.4 on fibroblasts from chick embryo heart, and also on a pure strain of sarcomatous fibroblasts of the rat.[1] The heteroproteose proved to be practically inactive, but both the alpha and beta proteoses caused a rapid proliferation of normal and sarcomatous fibroblasts. When tested at equal nitrogen concentrations, both the alpha and the beta proteoses produced approximately the same amount of tissue as the entire peptone solution (Text-fig. 1). The cells cultivated in alpha proteose, however, remained in better condition than those in beta proteose or peptone, and showed fewer of the granulations which are characteristic of fibroblasts cultivated in the hydrolytic products of proteins. Since Witte's peptone and the beta fraction contain some of the lower fragments of the protein molecule, it seems probable that these fragments are more toxic than the alpha fraction. Purification of the alpha proteose by repeated precipitation did not decrease its growth-promoting power for either normal or sarcomatous fibroblasts (Text-figs. 2, 3). The precipitations were repeated until a constant nitrogen concentration in three successive filtrates indicated, as pointed out by Haslam,[8] that all other fractions were removed. Peptic digests of fibrin were also fractionated into a part precipitated by hydrochloric acid at pH 5.0 and another part soluble at this acidity. Both fractions contained proteoses and

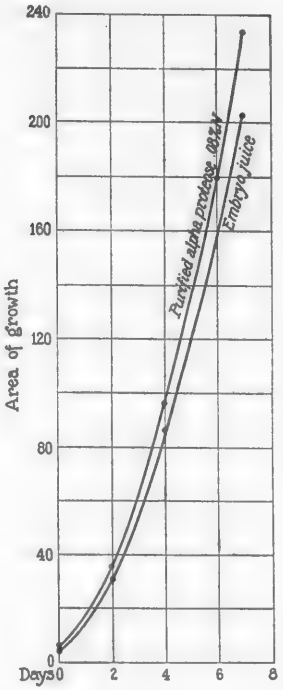
[6] For the technique of cultivating the tissues and making comparative experiments on the nutritive action of different media, see: Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503; Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 371; Carrel, A., *Compt. rend. Soc. Biol.*, 1927, xcvi, 601. In these experiments, the plasma coagulum was washed for 30 minutes with 3 cc. of Tyrode solution, shortly after coagulation took place.

[7] Fractionation of the proteoses into the proto- and deutero-fractions was not made, since such fractionation necessitates the use of salts. In previous experiments on the fractionation of embryo juice, the use of these salts always resulted in toxic or inactive products. Even when the greatest care was used in removing the salts, sufficient traces seemed to be left to exert a harmful effect on the cells.

[8] Haslam, H. C., *J. Physiol.*, 1905, xxxii, 267; 1907, xxxvii, 164.



TEXT-FIG. 1.



TEXT-FIG. 2.

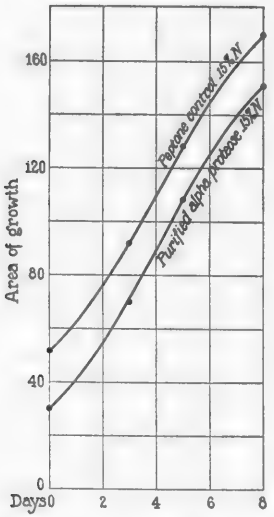
TEXT-FIG. 1. Experiment 9095-D. Comparative growth of fibroblasts from embryonic chick heart in alpha proteose, beta proteose,[9] and Witte's peptone at equal concentrations of nitrogen.

TEXT-FIG. 2. Experiment 9104-D. Comparative growth of fibroblasts from embryonic chick heart in purified alpha proteose,[9] and in embryo juice.

produced a large and approximately equal growth of sarcomatous and normal fibroblasts. It is evident, therefore, that the growth-promoting action of proteoses[9] is not a specific property of any one fraction,

[9] Although the proteoses produce a rapid growth for 8 or 10 days, they are not capable of supporting the life of the cells indefinitely. Other substances of unknown nature are required for the complete nutrition of the fibroblasts. A quantity of these substances sufficient for 8 or 10 days appears to be present already in the tissues. Salts and glucose are furnished by the Tyrode solution in which the proteoses are dissolved.

but that various fractions with different chemical properties are utilized by the cells.



TEXT-FIG. 3. Experiment 6149–C. Comparative growth of sarcomatous fibroblasts of the rat in Witte's peptone and purified alpha proteose.[9]

Action of the Peptones and Lower Protein Hydrolytic Products on Normal and Sarcomatous Fibroblasts.

The first attempts to ascertain whether the peptones, peptides, and amino acids are utilized by the cells were unsuccessful. Isolation of these fractions from Witte's peptone or protein digests, involving the use of such substances as salts, phosphotungstic acid, etc., resulted in inactive or toxic preparations. To avoid the use of injurious chemicals, the mixture of proteolytic products obtained by prolonged tryptic digestion was tried. Some growth occurred, but the cells degenerated rapidly. It was impossible to know whether the rapid degeneration was due to a toxic action of the lower protein degradation products or to the absence of other substances required to supplement their action. Moreover, pure strains of cells in the presence of hydrolytic products of protein underwent some change which caused the fibrin clot imme-

diately surrounding the colony to liquefy or digest. Thus, it seemed necessary to postpone further work on this subject until these difficulties could be overcome. Recently, the discovery of two facts has rendered possible the continuation of this investigation: (1) Pure strains of normal and sarcomatous fibroblasts of the rat can be cultivated in chicken plasma, even in the presence of protein split products, without liquefaction of the fibrin clot.[10] (2) Malignant fibroblasts from rat Sarcoma 10 of the Crocker Foundation will proliferate indefinitely in a peptic digest of liver.[11] While the chemical nature of all the substances in these liver digests which render possible the unlimited life of sarcomatous tissue *in vitro* is not yet known, it seemed probable that the use of liver hydrolyzed to various degrees would show whether products other than proteoses could be utilized by sarcomatous fibroblasts. Unfortunately, this method could not be applied as successfully to normal fibroblasts since, for some still unknown reason, they will not live indefinitely in the digests of liver. They proliferate rapidly at first, but accumulate large quantities of fat, multiply more slowly, and finally die.[12] In order that the substances of unknown nature which supplement the proteolytic products should not be removed, the various fractions were not isolated, but the total digest was used as a culture medium. By hydrolyzing liver with pepsin, trypsin, erepsin, and hydrochloric acid, and by modifying the concentration of enzymes and the hydrogen ion concentration, products were obtained in which the amount of proteoses varied from 0 to 50 per cent, and that of the nitrogen present as free amino groups from 19 to 81 per cent.

These experiments revealed that it is not only proteoses which furnish the nitrogenous materials required for growth, but that the lower split products can support the indefinite multiplication of the malignant fibroblasts from Sarcoma 10. While the destruction of proteoses by tryptic digestion decreased the growth of normal fibroblasts migrating from chick embryo heart,[2] the sarcomatous fibroblasts

[10] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 1119.

[11] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 371.

[12] For comparison of the growth of normal and sarcomatous fibroblasts in liver digests, see Text-fig. 3 in Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1928, xlviii, 105.

of the rat were found to multiply as rapidly and for as long a period in the tryptic digest of liver as in the peptic digest.

Effect of the Hydrolytic Products of Liver on Sarcomatous Fibroblasts.

The hydrolytic products of liver may be classified in four groups according to their action on sarcomatous fibroblasts. All were used at a nitrogen concentration of .03 to .04 per cent.

1. Peptic Digests in Which the Ratio of Total to Amino Nitrogen Was Above 2.8.—These digests contained 19 to 35 per cent of their nitrogen in the form of free amino groups and 40 to 50 per cent as proteose. When these substances were used, the rate of growth was slow, much less than in embryo juice or in the liver digests previously described.[11] The cells were scattered, very large, round and flat, and filled with many fatty globules. In fact, they seemed to be degenerating cells, although they kept these characteristics and continued to multiply slowly for about 6 weeks.[13]

2. Peptic Digests in Which Digestion Was More Complete.—35 per cent or more of the nitrogen was present as free amino groups, and 33 per cent or less as proteose. In these digests, the sarcomatous fibroblasts proliferated indefinitely and the cells remained in normal condition, like those cultivated in embryo juice.[14]

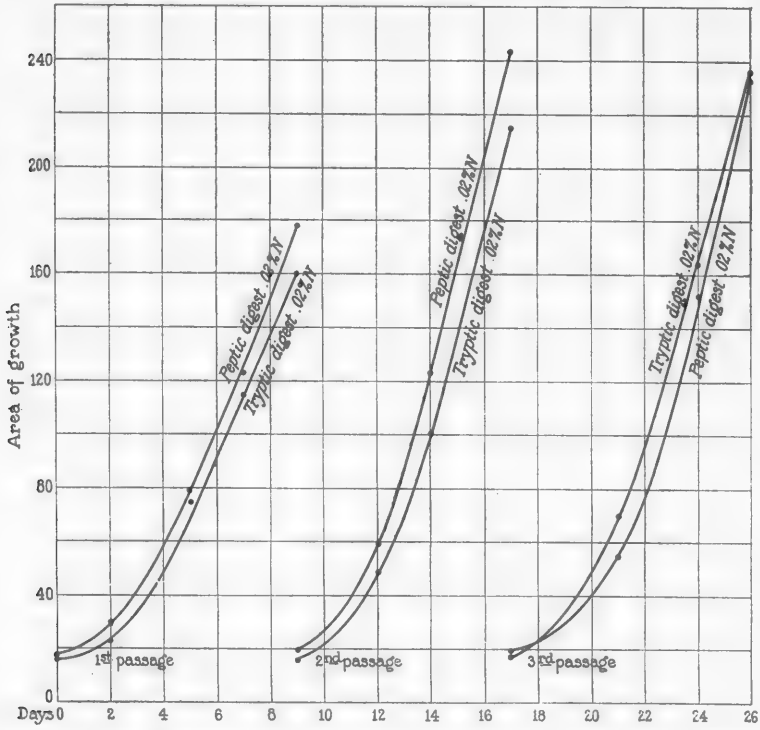
3. Tryptic Digests Hydrolyzed as Completely as Possible.—In these digests, the proteoses were entirely destroyed.[15] The ratio of total to amino nitrogen was 1.45, and 69 per cent of the nitrogen was present as free amino groups. Attempts were made to hydrolyze the remain-

[13] This conclusion was drawn from twenty-four experiments with seven different digests.

[14] This conclusion is the result of observations made during 1 year of colonies grown in many different preparations of this medium.

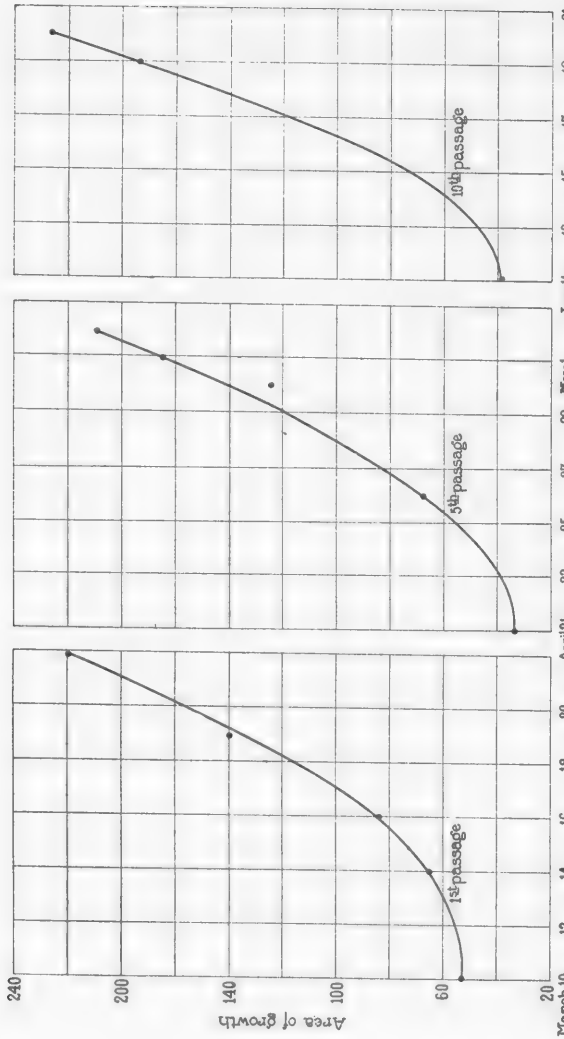
[15] When this preparation was tested for proteose by saturating at 33°C. with sodium sulfate, it was estimated that 3 per cent of proteose was present. However, since it is known that sodium and ammonium sulfate also bring down a small amount of the other proteolytic products, a control experiment was tried in which a mixture of pure amino acids and dipeptides was saturated with sodium sulfate at 33°C. Here also, 3 per cent of the nitrogen was precipitated. It is, therefore, concluded that this 3 per cent is not all proteose and, if any proteose remains, the quantity is negligible. At the dilution of this substance which was used in the culture medium, the quantity of proteose would be entirely without effect.

ing peptide linkings by further hydrolysis with enzymes, either trypsin or erepsin, but an equilibrium was always reached at which 13 per cent of the nitrogen remained in the peptide form. The remaining 18 per cent was in other forms which were not altered by boiling



TEXT-FIG. 4. Experiments 3987-A, 4020-A, and 4040-A. Comparative growth of sarcomatous fibroblasts of the rat for 26 days in the products of the peptic hydrolysis and complete tryptic hydrolysis of liver at equal nitrogen concentrations. Every 8 or 9 days, the colony was cut in half and transferred to a new flask.

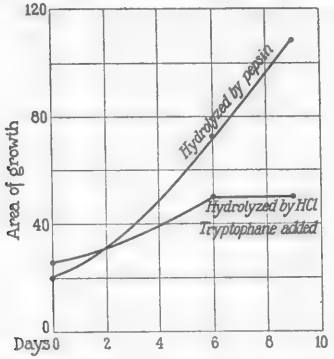
hydrochloric acid. In such media the sarcomatous fibroblasts proliferated indefinitely as rapidly as they did in embryo juice or in the peptic digests of Group 2 (Text-fig. 4). After the tissues had been cultivated for 102 days (Text-fig. 5), it was concluded that these preparations provided all the substances required for the growth of



TEXT-FIG. 5. Experiments 4289-A, 4478-A, and 4663-A. Growth of sarcomatous fibroblasts of the rat in the first, fifth, and tenth passages, or for 102 days, in the tryptic digest of liver containing 13 per cent of peptide nitrogen. The rate of growth was uniform throughout all the intermediate passages.

sarcomatous fibroblasts.[16] The cells differed in their appearance from those cultivated in embryo juice or peptic digests. They were small and oval shaped, and often grew in a sheet reminding one of epithelium. At the same time, the cytoplasm was filled with small fat droplets and numerous granules, and appeared less transparent than that of the cells cultivated in embryo juice. When transferred to embryo juice, the fibroblasts quickly became transparent again and contained fewer granulations.

4. Liver Completely Hydrolyzed by Boiling Hydrochloric Acid.—No growth occurred in these preparations, even when tryptophane was

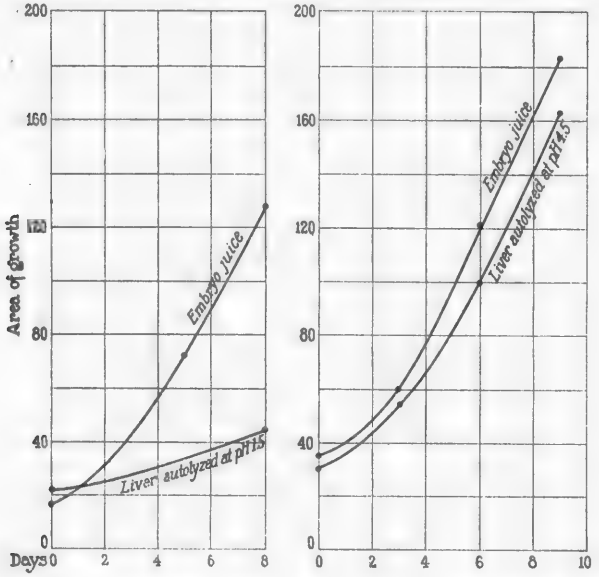


TEXT-FIG. 6. Experiment 4397-A. Comparatve growth of sarcomatous fibroblasts of the rat in the products of peptic hydrolysis of liver, and in the products of complete hydrolysis of liver by boiling hydrochloric acid. Tryptophane was added to replace that destroyed by the acid.

added to replace that destroyed by the acid (Text-fig. 6). These results are in accord with those of previously reported experiments[5] on the amino acids of embryo juice and artificial mixtures of amino acids. Although the amino acids present in the tryptic digests without doubt contribute to the growth of the tissue, and although glycocoll as such is utilized by them when added to albumin and casein digests, as shown in previous experiments,[3] amino acids alone have never been able to support cell proliferation. It appears that the 13 per cent of peptide nitrogen which remains in the tryptic digests is essential for the life of the cells *in vitro*.

[16] This conclusion was drawn from numerous experiments with four different preparations.

Several other preparations containing proteolytic products of liver have been tried. The results of these experiments agree with the preceding observations. Autolysis of liver at pH 1.5, the acidity used for preparing the peptic digests, resulted in a product of ratio 5:1, which resembled in its action the peptic digests insufficiently hydrolyzed. But autolysis at pH 4.5[17] gave a product of ratio 1:5, which caused rapid cell proliferation (Text-fig. 7), for a long period of time.



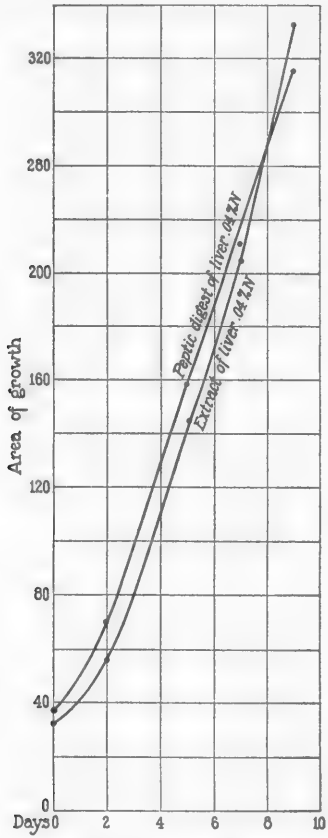
TEXT-FIG. 7. Experiments 4260-A and 4509-A. Comparative growth of sarcomatous fibroblasts of the rat in the products of liver autolysis at pH 1.5 and pH 4.5 with that in embryo juice.

The proteolytic products already present in liver were extracted with water at pH 9.0, and the proteins precipitated by adjusting to pH 5.0 and heating. This procedure was the same as the first steps taken by Cohn[18] in isolating from liver the substances effective in pernicious anemia. The resulting solution, like the second class of peptic

[17] A pH of 4.5 has been shown by Bradley to be the optimum for the activity of the autolytic enzymes of the liver. Bradley, H. C., *J. Biol. Chem.*, 1922, lii, 467.

[18] Cohn, E. J., Minot, G. R., Fulton, J. F., Ulrichs, H. F., Sargent, F. C., Weare, J. H., and Murphy, W. P., *J. Biol. Chem.*, 1927, lxxiv, p. lxix.

digests, had a ratio of total to amino nitrogen of 2:65, and proved capable of determining a prolonged growth of sarcomatous fibroblasts[19]



TEXT-FIG. 8. Experiment 10,173-D. Comparative growth of sarcomatous fibroblasts of the rat in Cohn's extract of liver and in the products of the peptic hydrolysis of liver at equal nitrogen concentration.

[19] The experiment was discontinued after 1 month. It is probably the proteolytic products already present in various organs which cause extracts of these organs to stimulate the multiplication of fibroblasts, as has been shown by Carrel, and Heaton. Carrel, A., *J. Exp. Med.*, 1913, xvii, 14; Heaton, T. B., *J. Path. and Bact.*, 1926, xxix, 293.

(Text-fig. 8). In fact, the autolytic products were in some respects better than the products of digestive enzymes, for the cells cultivated in such a medium were more transparent and similar to those maintained in embryo juice.

Fractionation with alcohol was tried in a few experiments. The constituents of a peptic digest soluble in 80 per cent alcohol produced for a considerable time as good a growth as the entire digest. The fraction soluble in 95 per cent alcohol failed to promote growth.

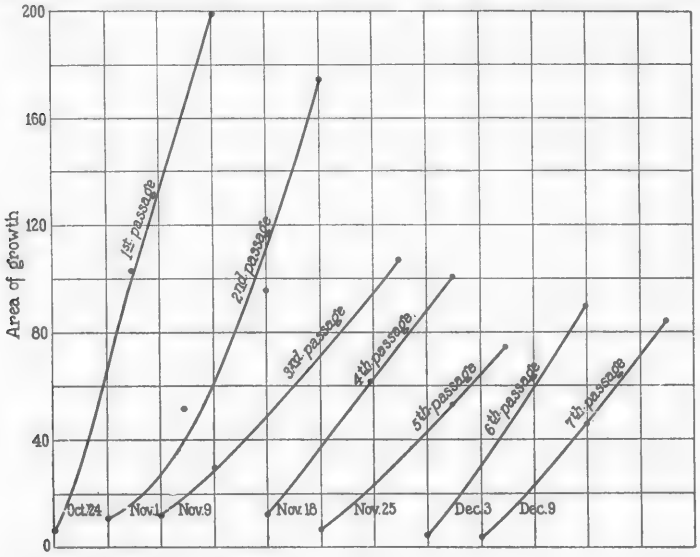
The above facts may be summarized as follows:

Products of the incomplete hydrolysis of the liver, either by pepsin or autolysis, that have less than 35 per cent of their nitrogen as free amino groups, do not support the growth of sarcomatous fibroblasts indefinitely. Peptic, autolytic, or tryptic digests, that have 35 to 69 per cent of their nitrogen as free amino groups, determine the unlimited proliferation of the cells. The active products are due to enzymatic action and are also preformed in the liver. Proteoses are utilized but are not required for growth. Peptones, peptides, and amino acids are also utilized. No growth, however, takes place in a medium in which all the peptide linkings are destroyed by acid hydrolysis. Although the acid may possibly act upon some other active group, there is little doubt that some peptides are required for the life of the cells.

Effects of the Hydrolytic Products of Liver on Normal Rat Fibroblasts.

Although normal cells do not proliferate indefinitely in liver digests, experiments of short duration were made with a view of comparing the behavior of normal and of sarcomatous fibroblasts. It was found that normal rat fibroblasts proliferated as rapidly for the first 5 or 6 days in the tryptic digests containing no proteose, as in the peptic digests. In both cases, the cells filled with fat, and growth decreased greatly in the second passage. The same was true in preparations of edestin and fibrin hydrolyzed to different degrees. It must be concluded, therefore, that normal cells also utilize the lower degradation products of protein. This does not contradict the previous observations[2] on the growth-activating effect of proteoses and

lower split fragments on fibroblasts from chick embryo heart, because pure strains of cells are more susceptible than fresh tissue to the toxic action or to the deficiencies of their medium, and because the hydrolytic products of liver used in these experiments, even those least hydrolyzed, always contained a large portion of the lower products. When these preparations were highly diluted so that they contained less than



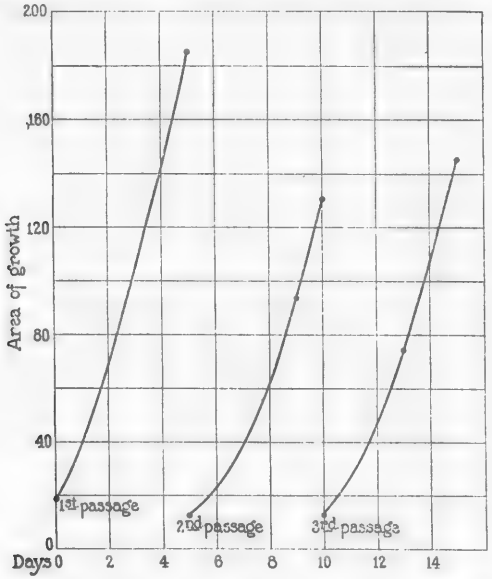
TEXT-FIG. 9. Experiment 3823-A. Growth of a pure strain of normal chicken fibroblasts in a peptic digest of liver. This digest was not boiled, but the toluene used during digestion was removed by aeration with nitrogen containing a small per cent of oxygen. Concentration of nitrogen, .02 per cent.

.02 per cent nitrogen, their toxic action was greatly reduced.[20] Normal fibroblasts could be kept alive in them for long periods of time without showing the characteristic, fatty degeneration deposits, and would multiply very slowly. In some of the proteolytic products of liver, pure strains of normal rat, and also of normal chicken, fibro-

[20] In the experiments previously reported, the concentration of proteolytic products in the media was much larger than that used in the present experiments. The large difference in growth shows that the lower fragments of the protein molecule are much more toxic to normal cells than the proteoses.

blasts were kept alive and growing very slowly for 2 months, at which time the experiment was discontinued (Text-figs. 9, 10). As the colonies had to be transferred to a new flask every 6 or 10 days, it is probable that some substance, possibly an enzyme present in the new plasma, supplemented the nutritive action of the liver digests.

The differences observed between the growth of the normal and sarcomatous rat fibroblasts in these media cannot as yet be explained.



TEXT-FIG. 10. Experiments 4391-A, 4416-A and 4440-A. Growth of normal fibroblasts of the rat in the alcohol-soluble fraction of Cohn's extract of liver. After the fourth passage, the rate of growth decreased markedly. Concentration of nitrogen, .0075 per cent.

It is evident that protein hydrolytic products including proteoses are more toxic to normal than to sarcoma cells. Since sarcoma cells, as shown by Warburg,[21] and observed also by us in pure cultures of sarcomatous fibroblasts,[22] possess a great glycolytic activity as compared with normal fibroblasts, the medium surrounding them is

[21] Warburg, O., *Biochem. Z.*, 1923, cxlii, 317; Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, clii, 309.

[22] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1928, xlviii, 105.

always acid. Its pH is approximately 6.0, while that of the medium surrounding normal fibroblasts is about 7.0. Between these concentrations of hydrogen ion the enzyme activity varies greatly. It seems quite plausible, therefore, that sarcoma cells synthetize protoplasm from peptides and peptones more readily than normal cells. As shown by Wasteneys and Borsook,[23] the synthesis of protein from proteolytic products of albumin by trypsin takes place readily at a hydrogen ion concentration varying from 5.5 to 6.5. It may equally well be that sarcoma cells have evolved enzymes which function somewhat differently from those of normal cells. Whatever the explanation of this difference, it is evident that sarcoma cells are able to proliferate rapidly and indefinitely on the products of enzymatic hydrolysis of proteins. This fact sheds further light on the mechanism of the growth of the tumor within the body. Any process bringing about protein digestion, such as takes place when dead or injured tissue is autolyzed by the enzymes set free or by macrophages, would supply sarcomatous fibroblasts with nutrient material.

CONCLUSIONS.

1. Both normal and sarcomatous fibroblasts of the rat utilize many different fragments of the protein molecule for their growth *in vitro*. Alpha and beta proteoses have approximately equal growth-promoting power.

2. A mixture of peptones, peptides, and amino acids, containing a negligible quantity of proteose, produces a temporary proliferation of normal fibroblasts, and an unlimited multiplication of sarcomatous fibroblasts, provided these substances are derived from liver which contains products of unknown nature that complete the nutritive effect of the protein degradation products.

3. Amino acids contribute to the nutrition of the cells, but are unable without the addition of peptides or polypeptides to support their life.

4. The proteolytic products are more toxic to normal than to sarcomatous fibroblasts. The hypothesis is suggested that the greater acidity produced by the large glycolysis of the sarcomatous cells may account for this difference through altering the speed of action of protein synthetizing enzymes.

[23] Wasteneys, H., and Borsook, H., *J. Biol. Chem.*, 1925, lxiii, 575.

BIOMETRY OF CALCIUM, INORGANIC PHOSPHORUS, CHOLESTEROL, AND LIPOID PHOSPHORUS IN THE BLOOD OF RABBITS.

I. Normal Animals from Recently Acquired Stock.

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The study presented by Brown (1) of calcium and inorganic phosphorus in the blood serum of normal rabbits showed certain definite results with respect to variation of these two elements over a given period of time. It seemed desirable to continue and extend these observations with a view to determining whether the variations that occurred followed a similar course from year to year and whether the changes in calcium and phosphorus were associated with corresponding variations in other constituents of the blood. In addition to calcium and inorganic phosphorus in the blood serum, two constituents of the whole blood were added, namely, cholesterol and lipoid phosphorus. The results obtained from these analyses of the blood are presented to show the degree of variation of each of these 4 constituents and a mathematical correlation of these variations. The experiments may be divided into two general groups: first, Group 1 to include all normal animals living under various environmental conditions; second, Group 2 to include diseased animals which have been inoculated either with *Treponema pallidum* or a malignant tumor. Each group under these two general classes will be presented separately.

Material and Methods.

The present discussion will be limited to those animals recently removed from outside living conditions, so that the results presented are, as nearly as possible, representative of an animal living out in the open.

From October 20, 1927, to May 15, 1928, determinations of calcium, inorganic phosphorus, cholesterol and lipoid phosphorus were made on 80 animals consisting of 8 groups of 10 each. The monthly determinations were made on a separate group of animals recently received by the laboratory from the breeding farms. The approximate age of the animals in each monthly group was between 6 and 8 months. In each instance, 10 animals were used, the mean values of calcium, inorganic phosphorus, cholesterol and lipoid phosphorus for the 10 animals determined, with their respective probable errors, the standard deviation from the means and the coefficients of variation. The curves for calcium, inorganic phosphorus, cholesterol and lipoid phosphorus in Fig. 1 were all plotted with the respective mean values given in mg. per 100 cc. Thus each monthly determination represents mean values for 10 animals, making a total of 80 animals examined over the period of 8 months. Except for the group of 10 animals examined on May 15, none of the animals were in the laboratory longer than 48 hours before the blood was examined, and in the last group the examination was made 72 hours after the animals were received in the laboratory.

On the day preceding examination, animals were fed as usual in the afternoon and were bled early the following morning before they had received additional food. The blood used for all determinations was drawn from the marginal ear vein of the rabbit. For cholesterol and lipoid phosphorus, 3 cc. of blood were drawn into a small test-tube into which had previously been placed 0.1 gm. sodium oxalate, the tube being constantly agitated until the total amount required was collected. Immediately after the blood for cholesterol and lipoid phosphorus was collected, 5 cc. of blood were drawn from the same opening into a graduated centrifuge tube and permitted to clot at room temperature. As soon as the clot was formed, it was separated from the side of the tube and centrifuged at 1400 revolutions per minute for 15 minutes. The lapse of time between the bleeding and the beginning of chemical analysis was never more than 2 hours, thus reducing to a minimum any error which might be due to the specimen of blood remaining too long at room temperature.

For the calcium and inorganic phosphorus the serum was measured with a 1 cc. tuberculin syringe graduated to 0.01 cc. and fitted with a 20 gauge needle. The whole blood for cholesterol and lipoid phosphorus was measured in like manner. Determinations of calcium and inorganic phosphorus were made on the blood serum according to the methods devised by Kramer and Tisdall (2) and Tisdall (3) respectively.

Cholesterol was determined by a slight modification of the method devised by Autenrieth (4), the modification consisting in the manner in which the cholesterol and lipoid phosphorus were extracted from whole blood; sodium oxalate in crystalline form being used as a non-coagulant. 1 cc. of whole blood was spread over a strip of fat-free filter paper 1½ by 7 inches. Two strips were used for each sample of blood, one for the determination of cholesterol and the other for the determination of lipoid phosphorus. These strips were then dried in an electric oven at 50°C.

The lipoid phosphorus was determined by the method described in a previous paper (5). The extraction of cholesterol with $CHCl_3$ was carried out in the same manner as described in the method for the determination of lipoid phosphorus. After the extraction with $CHCl_3$ was complete, the sugar tubes containing the cholesterol extract were placed in an electric oven at 100°C. until all the $CHCl_3$ had evaporated. The residue was taken up with small quantities of $CHCl_3$, transferred to a 15 cc. capacity Pyrex centrifuge tube and made up to 5 cc. 2 cc. acetic anhydride and 0.1 cc. H_2SO_4 were added and the color developed after standing 10 minutes was read against 5 cc. of a standard solution of cholesterol in a colorimeter. The standard solution of cholesterol was prepared by dissolving 20 mg. of cholesterol in 100 cc. of $CHCl_3$. 5 cc. of the standard solution are equal to 1 mg. cholesterol. In performing a series of duplicate analyses on the same sample of blood for the purpose of checking results and determining the degree of experimental error, the following average results were obtained: calcium 2.3 per cent, phosphorus 1.7 per cent, cholesterol 3.3 per cent and lipoid phosphorus 4.8 per cent variation in 100 cc. of sample.

RESULTS.

The results of analyses made in this experiment are presented in Text-figs. 1 to 7 and Tables I to VII. In all calculations, figures and tables, the lipoid phosphorus is calculated as lecithin, these calculations being made on the basis of results obtained by Levene (6) in which the phosphorus in lecithin is calculated as being approximately 4 per cent.

DISCUSSION AND CONCLUSIONS.

The following experiment was performed to demonstrate the variation of calcium in the same sample of blood serum when kept under two conditions of temperature.

30 cc. of blood were drawn by means of a hypodermic needle and syringe from the heart of a rabbit. The blood was divided into two portions of 15 cc. each and duplicate analyses for calcium were made on the serum immediately after centrifuging. The remaining portion of one sample was then corked and placed in the open laboratory; the other sample was corked and placed in the refrigerator at 4°C. The following are the results obtained:

Time after blood was drawn	Room temperature	Refrigerator
hrs.	*mg. per 100 cc.*	*mg. per 100 cc.*
0.5	13.4	13.6
1.5	13.0	13.6
24.0	12.6	13.6
52.0	13.2	14.2

TABLE I.

Values for Consecutive Monthly Determinations.

Date	Calcium			Inorganic phosphorus			Cholesterol			Lecithin		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
1927	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Oct. 20	18.5 ±0.394	3.59	19.41	6.045 ±0.173	0.811	13.42	51.1 ±1.188	5.57	10.90	94.8 ± 1.397	6.55	6.91
Nov. 17	16.1 ±0.158	0.740	4.59	5.230 ±0.190	0.890	17.01	64.5 ±1.89	8.89	13.78	135.8 ± 3.10	14.55	10.71
Dec. 15	14.7 ±0.156	0.733	4.98	6.811 ±0.220	1.06	15.69	68.7 ±2.94	13.79	20.08	100.3 ± 3.74	17.54	17.48
1928												
Jan. 12	14.5 ±0.100	0.469	3.23	6.109 ±0.190	0.890	14.58	73.3 ±1.342	6.29	8.58	125.1 ± 1.66	7.79	5.82
Feb. 21	15.8 ±0.180	0.845	5.38	4.960 ±0.204	0.958	19.31	71.2 ±1.85	8.69	12.21	168.3 ±10.18	47.75	23.85
Mar. 13	15.1 ±0.144	0.677	4.48	5.431 ±0.271	1.27	23.38	71.7 ±1.99	9.34	13.02	152.2 ± 2.75	12.88	8.46
Apr. 19	15.1 ±0.168	0.786	5.21	6.820 ±0.205	0.962	14.11	66.5 ±1.45	6.80	10.23	99.4 ± 2.50	13.83	13.91
May 15	14.5 ±0.162	0.760	5.24	5.245 ±0.167	0.785	14.97	67.6 ±2.293	10.75	15.90	118.6 ± 2.664	12.49	10.53

From the results of this experiment it may be seen that in the serum of the blood held at room temperature the calcium decreased 0.8 mg. per 100 cc. in 24 hours, while that held in the refrigerator was practically constant. 52 hours after the blood was drawn, both samples showed an increase in calcium of 0.6 mg. per 100 cc. from their preceding values. The sample held at room temperature was found

TABLE II.

Distribution of calcium		Distribution of calcium	
mg. per 100 cc.		*mg. per 100 cc.*	
12.5–13.4	2	19.5–20.4	0
13.5–14.4	23	20.5–21.4	2
14.5–15.4	25	21.5–22.4	1
15.5–16.4	18	22.5–23.4	0
16.5–17.4	4	23.5–24.4	1
17.5–18.4	2		
18.5–19.4	2	Observations.....80	

TABLE III.

Distribution of inorganic phosphorus	
mg. per 100 cc.	
3.00–3.99	3
4.00–4.99	15
5.00–5.99	24
6.00–6.99	26
7.00–7.99	9
8.00–8.99	3
Observations....80	

TABLE IV.

Distribution of cholesterol	
mg. per 100 cc.	
30–39.9	1
40–49.9	3
50–59.9	19
60–69.9	26
70–79.9	22
80–89.9	6
90–99.9	3
Observations..80	

TABLE V.

Distribution of lecithin	
mg. per 100 cc.	
70– 99.9	20
100–129.9	31
130–159.9	21
160–189.9	5
190–219.9	1
220–249.9	2
Observations....80	

to contain 1.0 mg. per 100 cc. less calcium than the sample held at 4°C. at the end of 52 hours.

Variation.—Fig. 1 is a graphic presentation of the results listed in Table I. An analysis of the results obtained for calcium shows the highest value of 18.5 ±0.36 mg. per 100 cc. of blood serum occurring in October. During November, December and January there occurred a gradual decrease in calcium from the above value until on

TABLE VI.

Coefficients of Correlation for Consecutive Monthly Determinations.

Date	rP. Ca	*P.E.*	rP. Chol.	*P.E.*	rP. Lec.	*P.E.*	rCa. Chol.	*P.E.*	rCa. Lec.	*P.E.*	rChol. Lec.	*P.E.*
1927												
Oct. 20	−0.444	±0.171	−0.133	±0.210	+0.031	±0.213	−0.295	±0.194	+0.205	±0.204	+0.341	±0.188
Nov. 17	−0.100	±0.211	+0.021	±0.213	−0.175	±0.207	+0.370	±0.184	+0.443	±0.171	+0.202	±0.205
Dec. 15	−0.438	±0.172	+0.519	±0.156	+0.029	±0.213	−0.170	±0.207	+0.162	±0.208	+0.002	±0.213
1928												
Jan. 12	−0.111	±0.211	+0.076	±0.212	+0.104	±0.211	+0.812	±0.073	−0.175	±0.207	−0.127	±0.210
Feb. 21	−0.709	±0.106	−0.404	±0.178	−0.201	±0.205	+0.115	±0.210	+0.250	±0.200	+0.112	±0.211
Mar. 13	−0.520	±0.156	+0.049	±0.213	+0.458	±0.169	−0.291	±0.195	−0.134	±0.209	+0.175	±0.207
Apr. 19	−0.423	±0.175	−0.220	±0.203	−0.118	±0.210	−0.220	±0.203	+0.495	±0.161	+0.099	±0.211
May 15	+0.476	±0.165	+0.404	±0.178	+0.302	±0.194	+0.495	±0.161	−0.045	±0.213	−0.118	±0.211

TABLE VII.

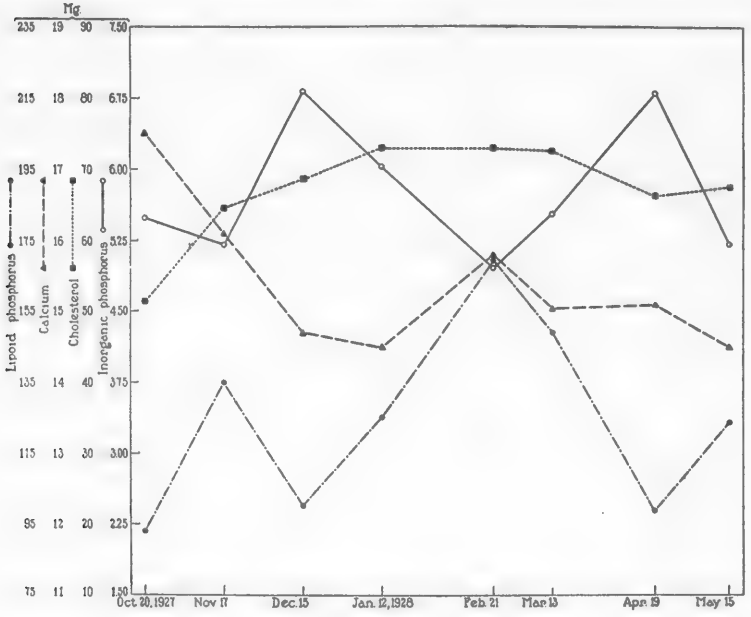
Section (a). Coefficients of Correlation of Group Means.

rP. Ca	*P.E.*	rP. Chol.	*P.E.*	rP. Lec.	*P.E.*	rCa. Chol.	*P.E.*	rCa. Lec.	*P.E.*	rChol. Lec.	*P.E.*
−0.094	±0.236	−0.137	±0.234	−0.794	±0.088	−0.887	±0.051	−0.235	±0.225	+0.560	±0.164

Section (b). Coefficients of Correlation with 80 Individual Values.

rP. Ca	*P.E.*	rP. Chol.	*P.E.*	rP. Lec.	*P.E.*	rCa. Chol.	*P.E.*	rCa. Lec.	*P.E.*	rChol. Lec.	*P.E.*
−0.146	±0.073	+0.002	±0.075	−0.347	±0.066	−0.340	±0.067	−0.088	±0.075	+0.285	±0.069

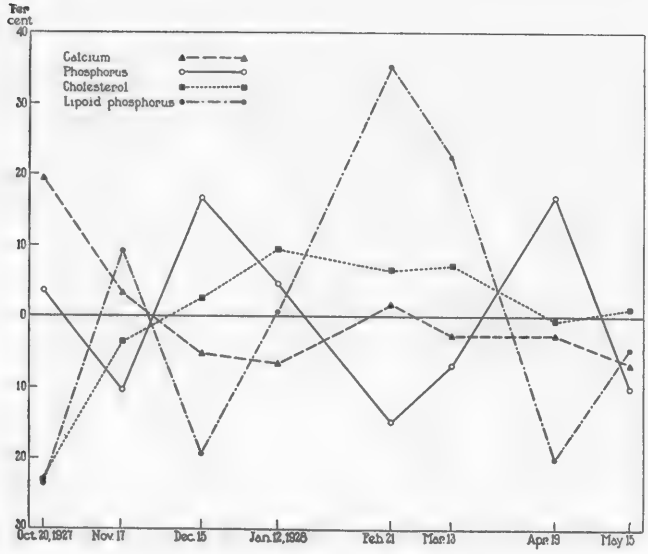
January 12, the amount of calcium was found to be 14.5 ±0.10. From January 12 to February 21, the calcium increased from 14.5 ±0.10 to 15.8 ±0.18 mg. per 100 cc. of blood serum. In February and through March, April and May the calcium gradually decreased in amount until on May 15 the mean value for the 10 animals was found to be 14.5 ±0.16. Thus the two extreme mean values for calcium during the 8 months of this experiment were found to be 18.5



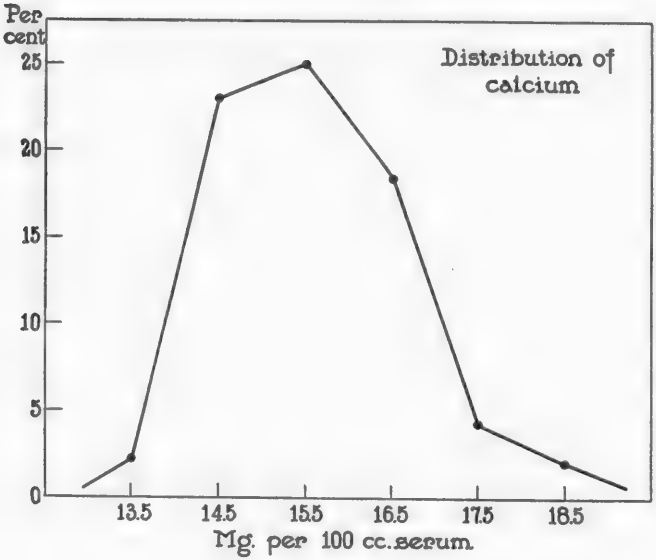
TEXT-FIG. 1.

±0.36 and 14.5 ±0.10 mg. per 100 cc. of blood serum. That this variation is of mathematic significance is demonstrated by the small probable error when compared with the difference between the two extremes. The results obtained are due to actual variations occurring in the blood serum and are not due to error of technique or chance sampling.

In actual mg. of the element, the variation of inorganic phosphorus

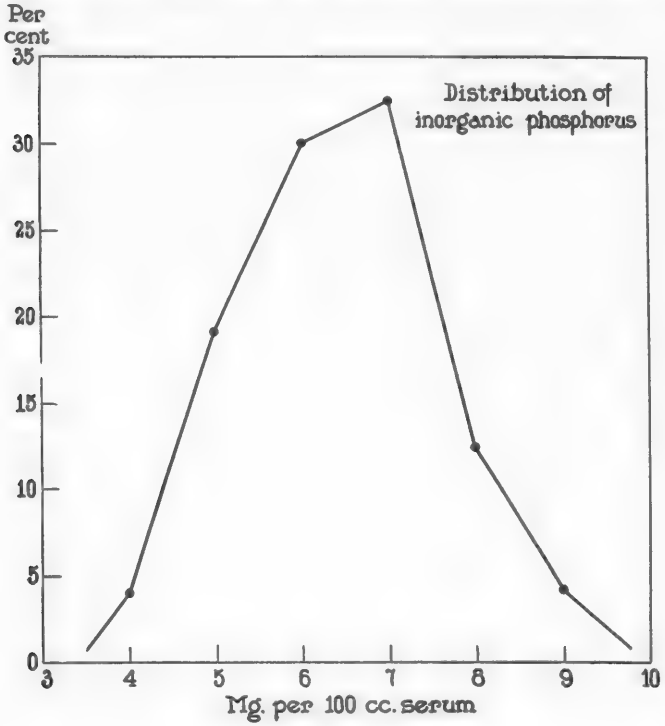


TEXT-FIG. 2.



TEXT-FIG. 3.

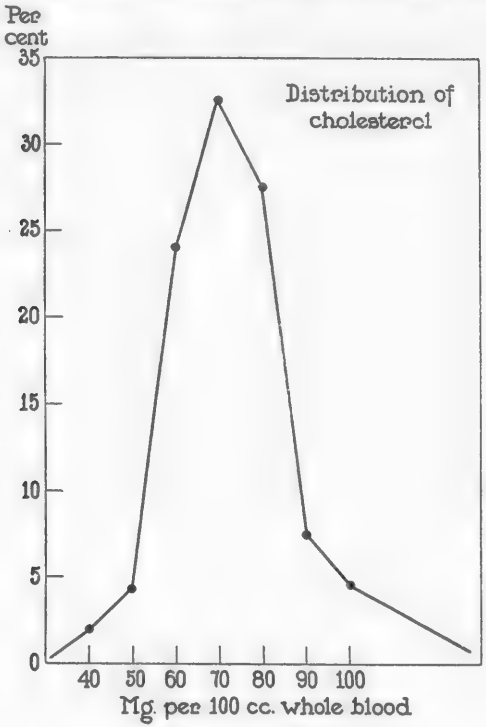
in the blood serum was found to be less than any one of the other 3 constituents. The frequency of fluctuation of phosphorus, however, was found to be greater than any one of the other constituents. When the mean value of 5.813 ±0.088 for phosphorus is considered, the variation of ±1.00 mg. occurring throughout the experiment is of high



TEXT-FIG. 4.

magnitude and can in no way be related to variation due to chance. From October to November, the inorganic phosphorus showed a decrease from 6.045 ±0.17 to 5.230 ±0.19 mg. per 100 cc. of blood serum. During December there was a marked increase reaching 6.811 ±0.22 on December 15. Through January and February the inorganic phosphorus decreased in amount until on February 21 the

mean value was found to be 4.960 ±0.20 mg. per 100 cc. of blood serum, this being the lowest value in the series of 8 monthly examinations. In March and April the inorganic phosphorus exhibited another marked increase similar to the increase first observed during November and December, reaching 6.820 ±0.20 mg. per 100 cc. of

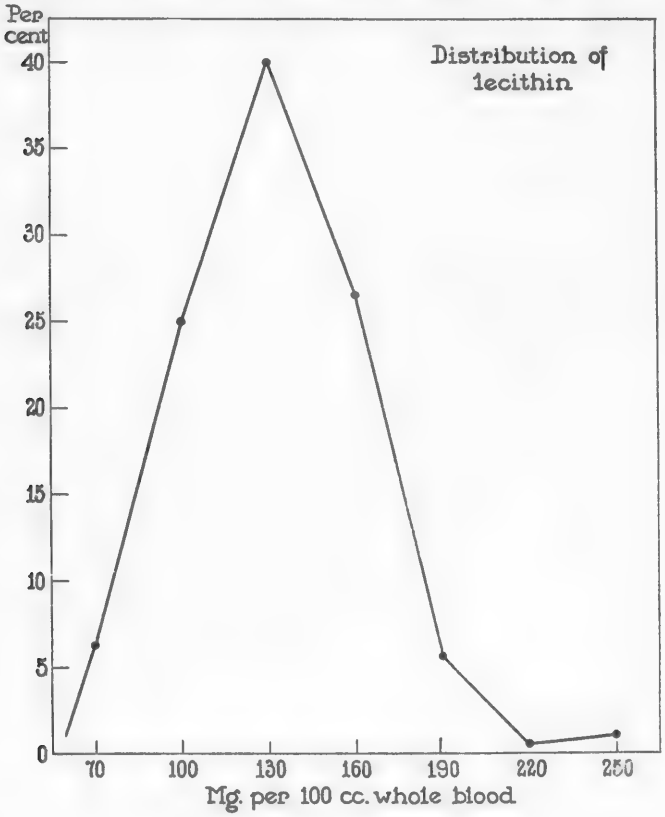


TEXT-FIG. 5.

blood serum on April 19. From this high value the trend was downward until on May 15 the amount of inorganic phosphorus was 5.245 ±0.16 mg. per 100 cc. of blood serum.

The stability of cholesterol was probably greater than any of the other 3 constituents. Beginning on October 20 with a mean value of 51.1 ±1.1 mg. per 100 cc. of whole blood, the cholesterol gradually

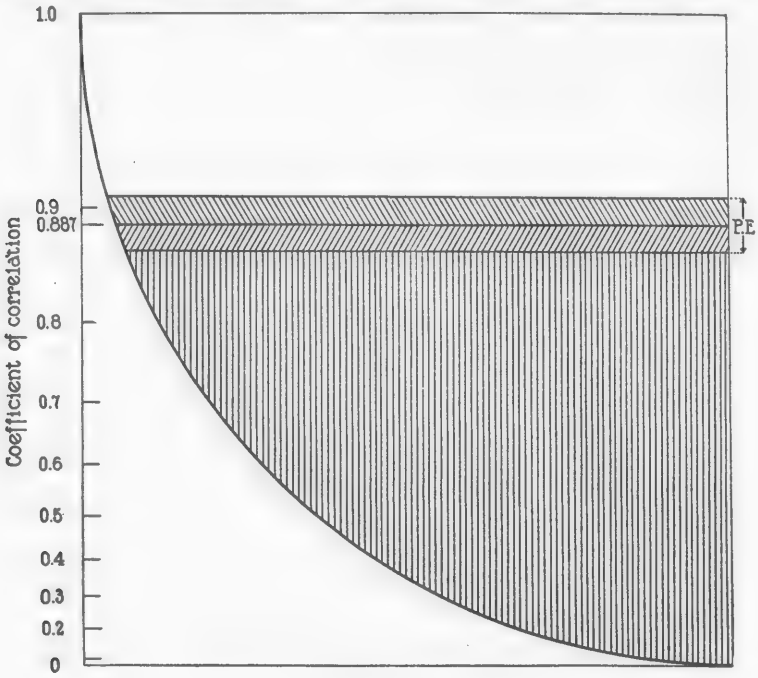
increased in amounts throughout November, December and on January 12 reached 73.3 ±1.3 mg. per 100 cc. of whole blood. During January, February and March the amounts of cholesterol found in the blood maintained a practically constant value. During March and



TEXT-FIG. 6.

April the cholesterol showed a slight decrease until on April 19 the mean value was found to be 66.5 ±1.4 mg. per 100 cc. of whole blood. The regularity of the curve for cholesterol suggests that this constituent of the whole blood shows the least degree of variation throughout the experiment.

Lipoid phosphorus, which in Fig. 1 is calculated and presented as lecithin, began with a value of 94.8 ±1.3 mg. per 100 cc. of whole blood. An increase occurred during October and November until on November 17 the lecithin was calculated to be 135 ±3.1 mg. per 100 cc. On December 15 the mean value for this group of animals was found to be 100.3 ±3.7, a marked decrease in amount of lecithin as compared



TEXT-FIG. 7.

with the previous monthly groups examined. During December and January there occurred a pronounced increase in lecithin until on February 21, 168.3 ±10.1 mg. per 100 cc. of whole blood was found to be present. The consequent decrease in lecithin during March and April reached its lowest level on April 19 at which time the lecithin in the whole blood was found to be 99.4 ±2.5 mg. per 100 cc. A slight increase occurred during the latter part of April and on May 15

the lecithin was found to be 118.6 ±1.6 mg. per 100 cc. of whole blood. Lecithin, when compared to the other 3 constituents, exhibited the greatest degree of variation. The difference between the two extreme values was 73.5 mg. or a fluctuation about its mean value of −29.7 and +33.8 mg. A comparison of the general average of calcium obtained in the present series of animals with the value for last year shows the following results.

	Results of Brown	Author's results
Calcium	15.4 ±0.09	15.5 ±0.14
Phosphorus	5.40 ±0.06	5.81 ±0.08

The trend of the per cent variation for each of the 4 constituents from their respective averages is presented in Fig. 2. The similarity between Figs. 1 and 2 is due to the fact that the divisions in mg. per 100 cc. in Fig. 1 were so calculated for calcium, inorganic phosphorus, cholesterol and lecithin that the variation of one was recorded in the same degree as any one of the other 3 constituents. The greatest extreme per cent variation occurred with lecithin, the values for this constituent of the whole blood ranging from +35 per cent to −20 per cent of the mean value 124.5 ±2.4. Inorganic phosphorus which in mg. per 100 cc. of serum had the least value gave a per cent variation from +18 to −15 per cent of the mean value 5.81 ±0.08. The per cent variation for calcium was from +20 to −5 per cent of the mean value 15.5 ±0.14. Cholesterol varied from +10 to −22 per cent of the mean value 66.8 ±0.84. While it is evident that these variations follow a seasonal trend, this subject will be discussed in a subsequent paper.

Averaging the 80 determinations made for each of the 4 constituents, the mean value for calcium was found to be 15.5 ±0.14; inorganic phosphorus 5.813 ±0.088 mg. per 100 cc. of blood serum; for cholesterol 66.8 ±0.846 and lecithin 124.5 ±2.47 mg. per 100 cc. of whole blood. Tables II, III, IV and V illustrated respectively with Figs. 3, 4, 5 and 6 refer to the distribution of calcium, inorganic phosphorus, cholesterol and lecithin about their respective mean values.

Correlation.—The trends of calcium, inorganic phosphorus, chol-

esterol and lecithin illustrated in Fig. 1 when taken for the entire 8 months, exhibit certain striking facts indicating possible relationships which may exist between these blood constituents. For example, the curve for inorganic phosphorus is almost a mirror image of the curve for lecithin; calcium and inorganic phosphorus show a similar relationship only to a less degree. With these facts in mind, Pearson's (7) formula for the measurement of the coefficient of correlation was applied to the 6 possible combinations of these 4 elements, that is, phosphorus × calcium, phosphorus × cholesterol, phosphorus × lecithin, calcium × cholesterol, calcium × lecithin and cholesterol × lecithin. Table VI gives the results with the probable errors obtained by calculating the correlation existing in each group of monthly determinations of 10 animals; Table VII gives the results with probable error obtained (*a*) with the mean values obtained for each monthly determination and (*b*) with the 80 animals used as one group. Section (*a*), therefore, represents the coefficient of correlation for the trend of the curves presented in Fig. 1.

Each of the results reported in Tables VI and VII, Sections (*a*) and (*b*), was calculated by the formula $r = \frac{\Sigma XY}{N \cdot \sigma X \cdot \sigma Y}$ in which

r = coefficient of correlation for each of the 6 possible combinations.
N = number of cases from which the mean value was derived.
ΣXY = the algebraic sum of the products of the deviations from the respective means for each series.
σ = standard deviations of the X and Y distributions respectively.

In order to obtain the value for ΣXY, the mean value of each of the 4 constituents is computed, that is, mean value for calcium, phosphorus, cholesterol and lecithin. The difference between this mean value and each single result is next calculated, maintaining, of course, the proper algebraic sign. Next the products of these differences for each combination, taking 2 constituents at a time are computed, that is, phosphorus × calcium, phosphorus × cholesterol, etc., maintaining throughout the proper sign. The Σ is then obtained by computing the algebraic sum of these product moments.

It is, therefore, quite evident from the above formula that unless there exists a difference between each separate determination and the mean value of the respective series, there can be no coefficient of correlation. For example, if 10 animals gave a mean value for calcium of 14.6 mg. per 100 cc. of serum and all the animals in the group had

the same value as the mean, the coefficient of correlation for that series with any one of the other constituents would be zero, due to the fact that the product moments of $X\ Y$ and the resulting $\Sigma\ X\ Y$ would be zero. In the results reported in Table VI, whatever factor or factors caused the values either to increase or to decrease, exerted its influence more or less equally on all the animals in the group so that the product moments and the resulting $\Sigma\ X\ Y$ was comparatively small. This fact is demonstrated by the results reported in Table VI in which the coefficient of correlation was calculated for each group of 10 animals.

In order to measure mathematically the trend of relationship of these 4 blood constituents throughout the period of 8 months, the coefficient of correlation was calculated by using the group means as presented in Fig. 1 and Table I. The results obtained from this calculation are presented in Table VII, Section (*a*) with their respective probable errors (*P.E.*). Of these 6 values, 3 stand out as being of mathematical significance, namely,

$$r \text{ phosphorus} \times \text{lecithin} = -0.794 \pm 0.088$$
$$r \text{ calcium} \times \text{cholesterol} = -0.887 \pm 0.051$$
$$r \text{ cholesterol} \times \text{lecithin} = +0.560 \pm 0.164$$

In each of the above results the coefficient of correlation is 3 times or more the probable error. If the coefficient is 3 times the probable error, the odds against the occurrence of a difference as great or greater than this in proportion to its probable error are 32 to 1, if chance alone were operative in the determination of the event. If we take the values of the coefficient of correlation for r phosphorus $\times$ lecithin and r calcium $\times$ cholesterol, the probabilities due to chance are even less than that calculated for r cholesterol $\times$ lecithin. Of the remaining 3 correlations, attention is called to the relation existing between calcium and phosphorus. While the sign of the coefficient in all cases except one is negative, the calculated probable error in this series does not permit a conclusive statement being made concerning this relationship.

With these values for the coefficient of correlation, the question arises as to what degree one may predict the trend of either constituent if one or the other is known. Fig. 7 illustrates the factor in per cent

above chance for the value obtained from r calcium $\times$ cholesterol. The shaded area, which is derived by drawing a line parallel to the abscissa through the value for the coefficient of correlation, represents a sector of a quadrant of a circle. The per cent of the total area of this sector as compared with the area of the whole quadrant is the factor above chance that calcium and cholesterol will continue in their stated relationship at the time of the next determination. With a coefficient of correlation for calcium $\times$ cholesterol of -0.887 ± 0.051, the probability in favor of the present trend continuing is 50.7 per cent above chance, or a 2 to 1 ratio.

The general average obtained for the 80 animals examined gave a value for inorganic phosphorus of 5.81 $\pm$0.08 mg. and 15.5 $\pm$0.14 mg. for calcium per 100 cc. of serum. For cholesterol the mean value was found to be 66.8 $\pm$ 0.84 mg. and for lecithin 124.5 mg. per 100 cc. of whole blood.

The coefficients of correlation calculated for the trend of these 4 blood constituents were found to be

r phosphorus $\times$ lecithin	$= -0.794 \pm 0.08$
r calcium $\times$ cholesterol	$= -0.887 \pm 0.05$
r cholesterol $\times$ lecithin	$= +0.560 \pm 0.16$
r phosphorus $\times$ calcium	$= -0.094 \pm 0.23$
r phosphorus $\times$ cholesterol	$= -0.137 \pm 0.23$
r calcium $\times$ lecithin	$= -0.235 \pm 0.22$

SUMMARY.

Determinations of calcium, inorganic phosphorus, cholesterol and lipoid phosphorus were made on a series of animals recently received from the dealer for the purpose of determining the trends of these 4 blood constituents throughout the year with the degree of their respective variations and mathematical correlation. It was found that for the 80 animals examined, calcium varied from 14.5 $\pm$0.10 to 18.5 $\pm$0.39 mg. and inorganic phosphorus 4.960 $\pm$0.20 to 6.820 $\pm$0.20 mg. per 100 cc. of blood serum. Cholesterol varied from 51.1 $\pm$1.18 to 73.3 $\pm$1.34 mg. and lecithin from 94.8 $\pm$1.397 to 168.3 $\pm$10.18 mg. per 100 cc. of whole blood.

Of the 6 possible combinations in calculating the coefficient of correlation for the trend throughout the experiment, 3 stand out as of

mathematical significance, namely the following. Between inorganic phosphorus and lecithin the coefficient of correlation was found to be -0.794 ± 0.088; between calcium and cholesterol -0.887 ± 0.051 and between cholesterol and lecithin $+0.560 \pm 0.164$.

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INFLUENCE OF LIGHT ENVIRONMENT ON THE ORGANIC CONSTITUTION OF NORMAL RABBITS WITH ESPECIAL REFERENCE TO THE ACTION OF NEON LIGHT.

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From observations made on normal rabbits, it was found that nutrition and growth and the growth of hair may be influenced by living in an environment of neon light, in the dark, or in an environment of diffuse, filtered sunlight of varying intensity, and that the effects produced by these conditions depend to some extent upon the color of the animal (1, 2).

When the observations on the rabbits used for these experiments had been completed, the animals were killed and their organs weighed for the purpose of determining whether any difference in the organic constitution of the several groups of animals could be detected, and whether a relation could be established between physical and functional states presented by animals living under different environmental conditions. The results of this phase of the experiments will be reported in the present paper.

Material and Methods.

The report is based on an analysis of the organ weights of 3 groups of normal rabbits. Each of the groups contained 15 animals, 5 of which had been living in an environment of neon light, 5 in the dark, and 5 received diffuse, filtered sunlight of varying intensity. Group I was composed of white, Group II of black, and Group III of gray, brown, and black animals.

The animals of Groups I and II were placed in their respective environmental conditions October 22, 1926, and remained under these conditions until they were killed on May 18 and 19, 1927, or for a period of approximately 7 months. The observations on the animals of Group III began October 1, 1926. On February 8, 1927, the light and dark divisions of this group were interchanged, so that

animals which had been living in an environment of neon light for approximately 4 months were placed in the dark and *vice versa*. This condition was maintained until May 4, 1927, when the two groups were restored to their original environments. The animals were killed June 8 and 9, or 5 weeks after the second change was made.

The organs were weighed in accordance with methods which have been described in detail elsewhere (3). The mean values obtained for actual weights of organs and for the weights per kilo of net body weight (relative weight) (3) are recorded in Table I.

For comparative purposes certain standard values are also given in Table I. These were derived from an analysis of body and organ weights of 645 normal rabbits (4). The standard mean for the weight of each organ is the mean value obtained for the 645 animals and represents the weight of organs for rabbits with a mean gross body weight of 2262 gm. or a net body weight of 1841 gm. The figures given in the first column of Table I as standard values are the values obtained for animals with a net body weight corresponding with that of the light, dark, or control animals (5).

The values obtained in these experiments are first compared with corresponding standard values; and then, a direct comparison is made of the results thus obtained for light, dark, or control animals of a given or of a different group. In order to reduce the standard value to a single expression, a mean net body weight for the 3 groups of animals of a given class (light, dark, control) was obtained and the standard values given in Table I for gross body weight and for organs are those for animals with a net body weight corresponding with these means.

Differences between the values obtained in these experiments and the standard values for normal rabbits are recorded in Table I as percentage deviations—positive (+) or negative (−). In this way, a correction is made for differences in the weights of organs which might be due to differences in the weights of animals, and at the same time, the results for all classes of animals are reduced to a common basis of expression which permits of a direct comparison. The results obtained for the relative weights of organs, as recorded in Table I, are presented graphically in Text-figs. 1 to 9.

No standard values were available for the lungs, so that the results given in the tables and text-figures were obtained by direct comparison of light and dark animals with the controls of the same group.

The percentage differences between light, dark, and control animals are summarized in Table II. The figures given for light and dark animals represent differences between animals of these classes and the controls of the same group; the difference between the light and dark animals is then found by a comparison of these two values.

RESULTS.

The results of the experiments are given in Tables I and II and Text-figs. 1 to 9.

TABLE I.

Actual and Relative Organ Weights in Gm. with Percentage Deviation from Standard Normal Values.

Organ	Standard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Group III
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Gross body weight		Standard mean [2262 gm.]					
Control	2796	2669	2740	2695	−4.54	−2.00	−3.61
Light	3044	2805	3110	2925	−7.85	+2.17	−3.91
Dark	2596	2455	2412	2715	−5.43	−7.09	−4.58
Gastrointestinal mass		Standard mean [419.9 gm.]					
Actual							
Control	432	417	408	410	−3.47	−5.56	−5.09
Light	504	404	455	445	−19.84	−9.72	−11.71
Dark	448	352	381	357	−21.43	−14.96	−20.31
Relative		Standard mean [232.2 gm.]					
Control	184	186	176	174	+1.09	−4.35	−5.43
Light	198	170	171	179	−14.14	−13.64	−9.60
Dark	208	169	189	153	−18.75	−9.14	−26.44
* Net body weight		Standard mean [1841 gm.]					
Control	2304	2296	2332	2285	− .35	+1.22	− .83
Light	2512	2401	2655	2480	−4.42	+5.69	−1.27
Dark	2164	2103	2031	2358	−2.82	−6.15	+8.97
Heart		Standard mean [5.28 gm.]					
Actual							
Control	6.86	6.50	7.03	6.02	−5.25	+2.48	−12.25
Light	6.51	6.84	7.17	6.81	+5.07	+10.14	+4.61
Dark	5.86	5.78	6.10	6.56	−1.37	+4.10	+11.95
Relative		Standard mean [2.87 gm.]					
Control	2.93	2.88	3.03	2.56	−1.71	+3.41	−12.63
Light	2.77	2.86	2.70	2.75	+3.25	−2.53	− .72
Dark	2.73	2.74	3.03	2.81	+ .37	+10.99	+2.93
Lungs—*cf.* Light and Dark with Control		Standard mean [ ]					
Actual							
Control		9.99	11.06	9.50			
Light		9.01	9.99	10.89	−9.81	−12.61	+13.91
Dark		8.97	8.26	10.75	−10.21	−28.03	+12.51
Relative		Standard mean [ ]					
Control		4.42	5.05	4.08			
Light		3.78	3.79	4.42	−14.48	−28.51	+7.69
Dark		4.27	3.97	4.57	−3.39	−24.43	+11.09

* The standard values for net body weight are the means for the 3 groups of animals of a given class.

TABLE I—*Continued.*

Organ	Standard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Group III
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Liver		Standard mean [85.8 gm.]					
Actual							
Control	86.9	87.0	76.6	69.6	+ .12	−11.9	−19.91
Light	90.5	74.0	83.0	78.6	−18.23	−8.29	−13.15
Dark	91.2	58.0	65.0	65.4	−36.40	−28.73	−28.29
Relative		Standard mean [47.4 gm.]					
Control	36.9	38.5	33.1	29.1	+4.34	−10.30	−21.14
Light	35.6	30.7	31.3	31.5	−13.76	−12.08	−11.52
Dark	42.5	27.5	32.1	28.0	−35.29	−24.47	−34.12
Kidneys		Standard mean [12.94 gm.]					
Actual							
Control	14.66	13.51	13.48	13.76	−7.84	−8.05	−6.14
Light	15.55	13.71	15.62	14.61	−11.83	+ .45	−6.05
Dark	13.79	11.89	11.87	12.13	−13.78	−13.92	−12.04
Relative		Standard mean [7.13 gm.]					
Control	6.25	5.98	5.80	5.86	−4.97	+2.48	+3.31
Light	6.56	5.68	5.94	5.91	−3.70	+3.20	+6.79
Dark	6.42	5.75	5.89	5.16	−2.23	+3.08	+11.05
Brain		Standard mean [9.17 gm.]					
Actual							
Control	9.66	9.18	9.90	9.98	−4.97	+2.48	+3.31
Light	10.01	9.64	10.33	10.69	−3.70	+3.20	+6.79
Dark	9.41	9.20	9.70	10.45	−2.23	+3.08	+11.05
Relative		Standard mean [5.08 gm.]					
Control	4.10	4.11	4.26	4.28	+ .24	+3.90	+4.39
Light	4.45	4.02	3.92	4.32	−9.66	−11.91	−2.92
Dark	4.38	4.38	4.79	4.46	± .00	+9.36	+1.83
Testicles		Standard mean [4.67 gm.]					
Actual							
Control	6.48	6.20	5.97	6.51	−3.32	−7.87	+ .46
Light	6.34	6.54	6.19	6.18	+3.16	−2.37	−2.52
Dark	5.48	5.56	4.93	5.35	+1.46	−10.04	−2.37
Relative		Standard mean [2.53 gm.]					
Control	2.76	2.77	2.56	2.82	+ .36	−7.25	+2.17
Light	2.77	2.74	2.33	2.51	−1.08	−15.88	−9.39
Dark	2.54	2.64	2.45	2.29	+3.94	−3.54	−9.84

TABLE I—*Continued.*

Organ	Standard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Group III
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Spleen		Standard mean [1.023 gm.]					
Actual							
Control	1.078	1.261	1.050	.976	+16.98	−2.60	−9.46
Light	1.334	1.071	.868	1.175	−19.64	−35.08	−11.92
Dark	1.140	.896	1.046	.908	−11.40	−8.25	−20.35
Relative		Standard mean [.546 gm.]					
Control	.458	.569	.454	.418	+24.24	− .87	−8.73
Light	.525	.449	.329	.475	−14.48	−37.33	−9.52
Dark	.530	.427	.538	.385	−19.43	+1.51	−27.36
Thymus		Standard mean [2.301 gm.]					
Actual							
Control	2.514	2.687	2.834	3.320	+6.96	+12.73	+22.06
Light	2.285	2.693	3.150	2.675	+17.86	+37.86	+17.07
Dark	2.526	2.737	2.503	2.878	+8.35	− .91	+13.94
Relative		Standard mean [1.264 gm.]					
Control	1.070	1.171	1.217	1.374	+9.44	+13.74	+28.41
Light	.897	1.123	1.223	1.081	+25.20	+36.34	+20.51
Dark	1.175	1.292	1.233	1.219	+9.96	+4.93	+3.75
Thyroid		Standard mean [.2328 gm.]					
Actual							
Control	.294	.301	.432	.4756	+2.28	+46.79	+61.60
Light	.373	.425	.881	.354	+13.85	+136.00	−5.17
Dark	.302	.316	.330	.3134	+4.60	+9.27	+3.74
Relative		Standard mean [.1265 gm.]					
Control	.1679	.1327	.185	.1881	−20.96	+10.19	+12.03
Light	.1579	.1756	.3345	.1413	+11.21	+111.84	−10.51
Dark	.1406	.1493	.1603	.1322	+6.19	+14.01	−5.97
Parathyroids		Standard mean [.01286 gm.]					
Actual							
Control	.0125	.0135	.0146	.0138	+8.00	+16.80	+10.40
Light	.0172	.0162	.0146	.0148	−5.81	−15.12	−13.95
Dark	.0139	.0129	.0140	.0182	−7.19	+ .72	+30.91
Relative		Standard mean [.00709 gm.]					
Control	.0053	.00592	.00626	.00587	+11.70	+18.11	+10.76
Light	.0075	.00667	.00553	.00597	−11.07	−26.27	−20.40
Dark	.0065	.00616	.00707	.00781	−5.23	+8.77	+20.00

TABLE I—*Continued.*

Organ	Standard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Group III
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Hypophysis		Standard mean [.0281 gm.]					
Actual							
Control	.031	.026	.031	.0284	−16.13	± .00	−8.39
Light	.0335	.0274	.0344	.0326	−18.21	+2.69	−2.69
Dark	.0303	.026	.028	.0262	−14.19	−7.59	−13.53
Relative		Standard mean [.0155 gm.]					
Control	.0133	.01137	.01338	.01243	−14.51	+ .60	−6.54
Light	.0139	.01137	.01312	.0131	−18.31	−5.61	−5.76
Dark	.01418	.01245	.01396	.0113	−12.20	−15.52	−19.75
Suprarenals		Standard mean [.3833 gm.]					
Actual							
Control	.559	.461	.664	.631	−17.53	+18.78	+12.88
Light	.583	.479	.640	.4752	−17.84	+9.78	−18.49
Dark	.427	.482	.529	.511	+12.88	+23.89	+19.67
Relative		Standard mean [.2082 gm.]					
Control	.238	.209	.290	.2705	−11.76	+21.85	+13.66
Light	.254	.1997	.2455	.1911	−21.38	−3.35	−24.46
Dark	.197	.2318	.2635	.2135	+17.67	+33.76	+8.38
Pineal		Standard mean [.01577 gm.]					
Actual							
Control	.0166	.016	.0174	.016	−3.61	+4.82	−3.61
Light	.0189	.017	.017	.0132	−10.05	−10.05	−30.16
Dark	.0164	.0156	.014	.0182	−4.88	−14.63	+10.98
Relative		Standard mean [.00871 gm.]					
Control	.0071	.00602	.00749	.00706	−15.21	+5.49	− .56
Light	.0074	.00665	.00618	.00534	−10.14	−16.49	−27.84
Dark	.0077	.00824	.00694	.00775	+7.01	−9.87	+ .65
Axillary lymph nodes		Standard mean [.1697 gm.]					
Actual							
Control	.160	.133	.112	.0976	−16.88	−30.00	−39.00
Light	.129	.157	.145	.1086	+20.16	+12.40	−15.00
Dark	.156	.148	.132	.0850	−5.13	−15.38	−45.51
Relative		Standard mean [.09368 gm.]					
Control	.0679	.0594	.0486	.0425	−12.52	−28.42	−37.41
Light	.0511	.0655	.0547	.0438	+28.18	+7.05	−14.29
Dark	.0728	.0701	.0658	.0350	−3.71	−9.62	−51.92

TABLE I—*Concluded.*

Organ	Standard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Group III
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Popliteal lymph nodes		Standard mean [.2557 gm.]					
Actual							
Control	.251	.220	.207	.214	−12.36	−17.53	−14.74
Light	.223	.213	.223	.243	−4.48	± .00	+9.06
Dark	.244	.194	.203	.188	−20.33	−16.89	−23.03
Relative		Standard mean [.14066 gm.]					
Control	.1068	.0974	.0894	.0910	−8.80	−16.29	−14.81
Light	.0878	.0884	.0847	.0981	+ .68	−3.53	+11.73
Dark	.1135	.0952	.1021	.0792	−16.30	−10.04	−30.26
Mesenteric lymph nodes		Standard mean [3.46 gm.]					
Actual							
Control	4.18	3.42	2.14	2.49	−18.18	−48.80	−40.43
Light	4.42	2.81	2.30	2.58	−36.42	−47.96	−41.66
Dark	3.60	2.30	2.08	2.28	−36.11	−42.22	−36.67
Relative		Standard mean [1.885 gm.]					
Control	1.69	1.508	.917	1.09	−10.77	−45.74	−35.50
Light	1.74	1.187	.850	1.04	−31.78	−51.15	−40.23
Dark	1.68	1.090	1.020	.97	−35.12	−33.33	−42.26
Deep cervical lymph nodes		Standard mean [.1563 gm.]					
Actual							
Control	.178	.144	.214	.206	−19.10	+20.22	+15.73
Light	.166	.132	.217	.135	−20.48	+30.72	−18.67
Dark	.175	.146	.096	.208	−14.86	−45.03	+18.63
Relative		Standard mean [.0847 gm.]					
Control	.0756	.0664	.0926	.0901	−12.17	+22.49	+19.18
Light	.0656	.0564	.0850	.0542	−14.02	+29.57	−17.38
Dark	.0817	.0703	.0473	.0851	−13.97	−42.11	+4.16

TABLE II.

Difference in Per Cent between Control, Light, and Dark Divisions of the Same and of Different Groups.

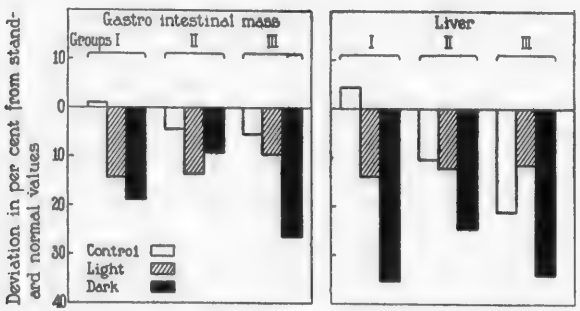
Organ	Actual weight			Relative weight		
	Group I	Group II	Group III	Group I	Group II	Group III
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Gross body weight						
Light	−3.31	+4.17	− .30			
Dark	+ .89	−5.09	− .97			
Difference	4.20	9.26	.67			
Net body weight						
Light	−4.07	+4.47	− .44			
Dark	−2.47	−4.93	+8.14			
Difference	1.60	9.40	8.58			
Gastrointestinal mass						
Light	−16.37	−4.16	−6.62	−15.23	−9.29	−4.17
Dark	−17.96	−9.40	−15.22	−19.84	−4.79	−21.01
Difference	1.59	5.24	8.60	4.61	4.50	16.84
Heart						
Light	+10.32	+7.66	+16.86	+4.69	−5.94	+11.91
Dark	+3.88	+1.62	+24.20	+2.08	+7.58	+15.56
Difference	6.44	6.04	7.34	2.61	13.52	3.65
Lungs						
Light	−9.81	−12.61	+13.91	−14.48	−28.51	+7.69
Dark	−10.21	−28.03	+12.51	−3.39	−24.43	+11.09
Difference	.40	15.42	1.40	11.09	4.08	3.40
Liver						
Light	−18.35	+3.61	+6.76	−18.10	−1.78	+9.62
Dark	−36.52	−20.41	−8.38	−39.63	−14.17	−12.98
Difference	18.17	24.02	15.14	21.53	12.39	22.60
Kidneys						
Light	−3.99	+8.50	+ .09	+1.27	+ .72	+3.48
Dark	−5.94	−5.87	−5.90	+2.74	+ .60	+7.74
Difference	1.95	14.37	5.81	1.47	.12	4.26
Brain						
Light	+1.27	−9.90	+ .72	−15.81	+3.48	−7.31
Dark	+2.74	− .24	+ .60	+5.46	+7.74	−2.56
Difference	1.47	9.66	.12	21.27	4.26	4.75

TABLE II—*Continued.*

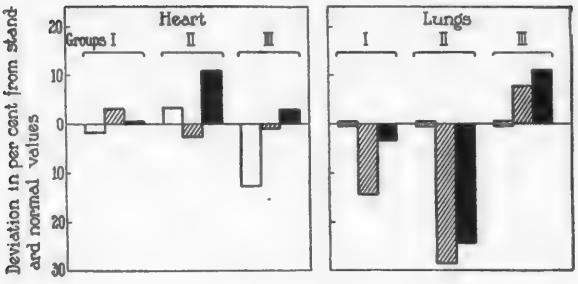
Organ	Actual weight			Relative weight		
	Group I	Group II	Group III	Group I	Group II	Group III
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Testicles						
Light	+6.48	+5.50	−2.98	−1.44	−8.63	−11.56
Dark	+4.78	−2.17	−2.83	+3.58	+3.71	−12.01
Difference	1.70	7.67	.15	5.02	12.34	.45
Spleen						
Light	−36.62	−32.48	−2.46	−38.72	−36.46	− .79
Dark	−28.38	−5.65	−10.89	−43.67	+2.38	−18.63
Difference	8.24	26.83	8.43	4.95	38.84	17.84
Thymus						
Light	+10.90	+25.13	−4.99	+15.76	+22.60	−7.90
Dark	+1.39	−13.64	−8.12	+ .52	−8.81	−24.66
Difference	9.51	38.77	3.13	15.24	31.41	16.76
Thyroid						
Light	+11.57	+89.21	−66.77	+32.17	+101.65	−22.54
Dark	+2.32	−37.52	−57.86	+27.15	+3.82	−18.00
Difference	9.25	126.73	8.91	5.02	97.83	4.54
Parathyroids						
Light	−13.81	−31.92	−24.35	−22.77	−44.38	−31.16
Dark	−15.19	−16.08	+30.91	−16.93	−9.34	+20.00
Difference	1.38	15.84	55.26	5.84	35.04	51.16
Hypophysis						
Light	−2.08	+2.69	+5.70	−3.62	−6.31	+ .78
Dark	+1.94	−7.59	−5.14	+2.31	−16.12	−13.21
Difference	4.02	10.28	10.84	5.93	9.81	13.99
Suprarenals						
Light	− .31	−9.00	−31.37	−9.62	−25.20	−38.12
Dark	+30.41	+5.11	+6.79	+29.43	+11.91	−5.28
Difference	30.72	14.11	38.16	39.05	37.11	32.84
Pineal						
Light	−6.44	−14.87	−26.55	+5.07	−21.98	−27.28
Dark	−1.27	−19.45	+14.59	+22.22	−15.36	+1.21
Difference	5.17	4.58	41.14	17.15	6.62	28.49

TABLE II—*Concluded.*

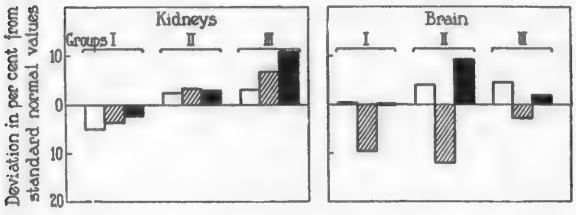
Organ	Actual weight			Relative weight		
	Group I	Group II	Group III	Group I	Group II	Group III
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Axillary lymph nodes						
Light	+37.04	+42.40	+24.00	+40.70	+35.47	+23.12
Dark	+11.75	+14.62	−6.51	+8.81	+18.80	−14.51
Difference	25.29	27.78	30.51	31.89	16.67	37.63
Popliteal lymph nodes						
Light	+7.88	+17.53	+23.80	+9.48	+12.76	+26.54
Dark	−7.97	+ .64	−8.29	−7.50	+6.25	−15.45
Difference	15.85	16.89	32.09	16.98	6.51	41.99
Mesenteric lymph nodes						
Light	−18.24	+ .84	−1.23	−21.01	−5.41	−4.73
Dark	−17.93	+5.74	+3.76	−24.35	+12.41	−6.76
Difference	.31	4.90	4.99	3.34	17.82	2.03
Deep cervical lymph nodes						
Light	−1.38	+10.50	−34.40	−1.85	+7.08	−36.56
Dark	+4.24	−65.25	+2.90	−1.80	−64.60	−15.02
Difference	5.62	75.75	37.30	.05	71.68	21.54



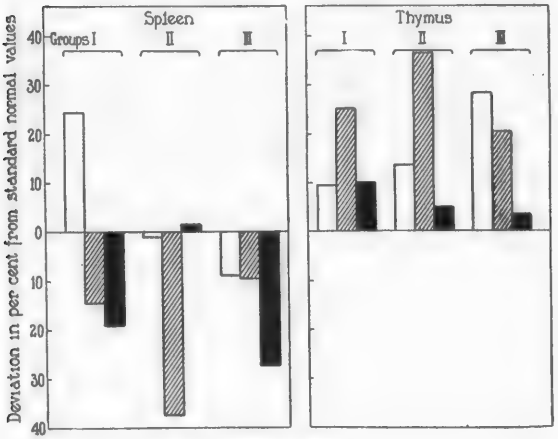
TEXT-FIG. 1.



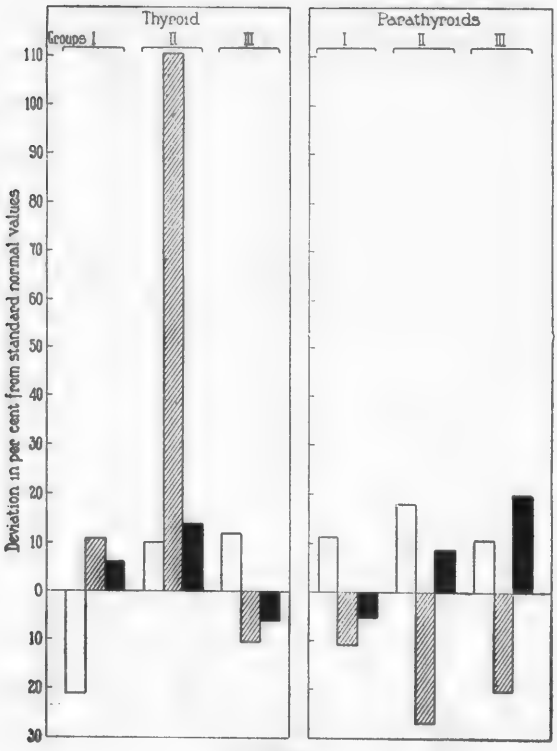
TEXT-FIG. 2.



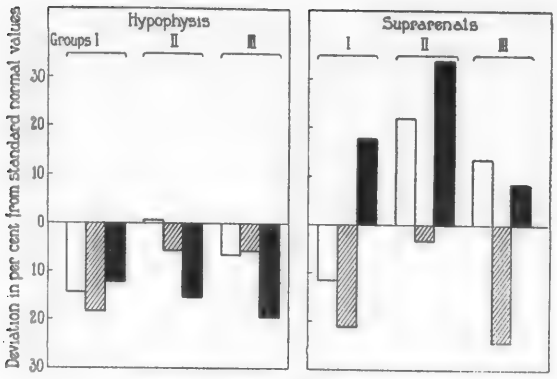
TEXT-FIG. 3.



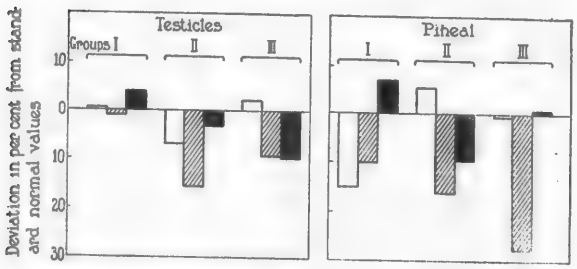
TEXT-FIG. 4.


Thyroid
Parathyroids
Groups I
II
III
I
II
III
Deviation in per cent from standard normal values
110
100
90
80
70
60
50
40
30
20
10
0
10
20
30

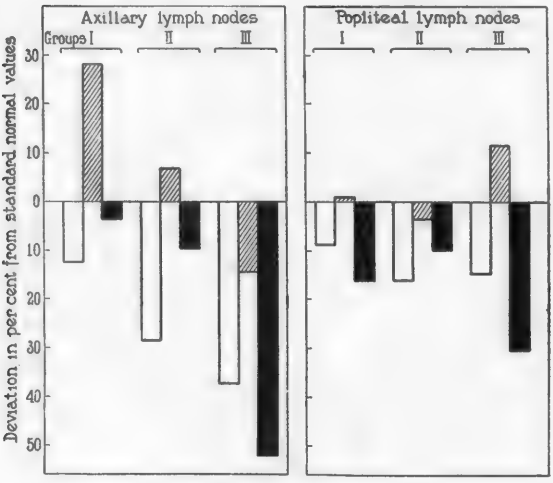
TEXT-FIG. 5.



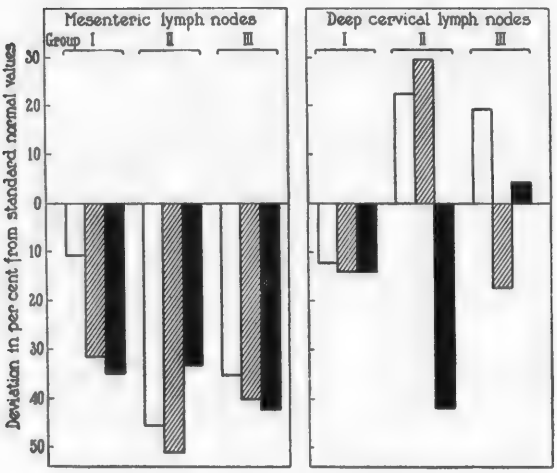
TEXT-FIG. 6.



TEXT-FIG. 7.



TEXT-FIG. 8.



TEXT-FIG. 9.

DISCUSSION AND CONCLUSIONS.

In comparing the experimental results with standard values, some allowance must be made for inherent differences in material. The standard values represent a cross-section of the material used for ordinary experimental purposes in this laboratory over a period of $3\frac{1}{2}$ years, and this material included animals of various breeds, ages, and weights, but was composed mainly of gray and brown rabbits, 8 to 12 months old, which had been caged in the laboratory for 2 to 8 weeks. The animals of the present series were of a comparable age at the beginning of the experiments, but they had been caged in the laboratory approximately 8 months. Group I was composed entirely of white rabbits, Group II of black, while Group III contained animals of a dark color. The age difference is partially, if not entirely, compensated by the correction made for weight with the exception of the dark animals of Groups I and II which were undoubtedly older than the average animal of corresponding weight in the standard series.

A further distinction is to be made on the basis of the occurrence of periodic or cyclic variations in the weights of organs (6). The standard material was collected over a period of $3\frac{1}{2}$ years and included a wide range of spontaneous variations, while the experimental results represent values obtained at a particular time. In this connection, it should also be pointed out that some difference may be expected between the results for Groups I and II on the one hand and Group III on the other, due to the fact that the animals were killed at a time when, as a rule, the weights of organs are changing, and that there was an interval of 3 weeks between the killing of the first and last groups.

Considering the results recorded above, the points to be determined are, first, whether the results obtained show any material difference in the physical constitution of animals living under different environmental conditions, and, second, whether there is any evidence that the effects produced were influenced by the color or breed of the animals. In analyzing the results, Groups I and II are to be regarded as representing comparable environmental conditions; the status of Group III is problematic as the light and dark divisions of this group were interchanged twice during the experiment, and these animals were

returned to their original environmental conditions only 5 weeks before the experiment was concluded. The question is, therefore, whether the results obtained suggest a conformity with animals of the same class in Groups I and II or a reverse relation.

By reference to Tables I and II and Text-figs. 1 to 9, it will be seen that in most instances, the values obtained for light, dark, and control animals tend to preserve a common relation to the standard values, irrespective of differences in environmental conditions. It is evident, therefore, that the results are affected by the action of some factor which influences the weights of organs of all animals in a given manner, but not necessarily to the same degree. For example, the actual and relative weights of the gastrointestinal tract were subnormal for all except one lot of control animals (Tables I and II and Text-fig. 1). This is an almost constant finding for rabbits that have been caged in the laboratory for considerable periods of time. From a further analysis of the results, it will be seen, however, that in all cases the weights for light and dark animals were smaller than those for the corresponding controls (Text-fig. 1). The control animals of Groups I, II, and III showed a progressive decrease in the relative weight of the gastrointestinal mass, and the light groups a progressive increase, while the results for the 3 groups of animals in the dark were irregular.

A similar result was obtained for the liver (Text-fig. 1) as might be expected on account of the functional relation between the two organs. The progressive reduction in the weight of the liver of control animals was greater than that of the gastrointestinal mass, while the values for animals in the light remained virtually constant. Otherwise the results show an absolute agreement which is made particularly striking by the fact that the percentage values for light animals are practically the same for gastrointestinal mass and liver.

The results for the heart are irregular and show no clearly defined difference. There is merely a suggestion that the weight of the heart per kilo of body weight may have been greater for animals in the dark than for control and light groups (Text-fig. 2).

The significance of the results for the lungs is also uncertain. The weights for light and dark animals of Groups I and II were less than the controls but were greater for Group III. There is, however, a

consistent relation between the values for light and dark animals in that the value for animals in the dark is larger in all cases than that for animals in the light (Text-fig. 2).

The relative weights of the kidneys (Text-fig. 3) furnish an interesting example of an instance in which there is a remarkably close agreement between all values for a given group and only a slight difference between experimental and standard values with an extremely small but consistent difference between light and dark animals and the corresponding controls. The difference in weight is not sufficient in itself to be regarded as of any significance but the fact that the values for light and dark animals are larger than those for the controls in all cases and that those for animals in the dark tend to be larger than those for the light leads one to suspect that the results may be significant. Attention may also be called to the fact that while all values for white animals are below normal, those for dark colored animals are above normal.

The results for the brain are of a similar order but with differences that are more clearly defined (Table I and Text-fig. 3). In the case of actual weights, the relative order of magnitude is control, light, and dark, but when the results are reduced to weights per kilo of body weight, the values for both control and dark animals are at or above normal, while those for light animals are below normal with a clear line of separation between. In this case, there is a further suggestion of a difference between white and black animals on the one hand, and Groups II and III on the other. This is seen chiefly in the results for the control and dark divisions of Groups I, II, and III and in the reduction of the deviation from normal of both the light and dark divisions of Group III as compared with Group II, while the controls remain constant (Text-fig. 3).

The values obtained for the actual weight of the spleen (Table I) show that this organ was distinctly larger in control than in light and dark animals. There is also evidence that it was larger in white than in animals of dark color. The smallest values for Groups I and II were given by animals living in the light. In Group III, the smallest value was given by animals of the dark division. But if we consider the order of magnitude of the values for light and dark divisions of the 3 groups, there is a suggestion that the figures for Group III show a

reversed relation due to the persistence of an effect which was not abolished during the last 5 weeks of the experiment. The results for relative weight (Text-fig. 4) show essentially the same relations, but they are not brought out so clearly as they are by a comparison of actual weights.

The conditions shown by the thymus are just the reverse of those shown by the spleen in so far as the relative magnitudes of the values for different classes of animals are concerned. This is, of course, to be expected on account of the relation that obtains between these two organs.

The results for the thyroid are very striking (Table I and Text-fig. 5). In the first place, the results for control animals show that there is unquestionably a difference in the weight of the thyroid of white and of black or dark colored animals. This difference holds also for the light and dark divisions of Groups I and II, but not for Group III. In the second place, the animals of the light divisions of Groups I and II gave the highest values and the controls the lowest, while in Group III this relation was reversed. The very high value obtained for the light animals of Group II represents a condition shown by 4 of the 5 rabbits comprising this division and is, therefore, a valid result. The reduction in the weight of the thyroid shown by the light and dark divisions of Group III is difficult to account for, but may be a result of recent changes in environmental conditions.

The parathyroids show a clear separation according to environmental conditions. Actual and relative weights for animals in the light are all subnormal and smaller than those for the corresponding dark divisions, while the values for the controls are above normal and in 2 of the 3 groups are larger than those for dark animals (Tables I and II and Text-fig. 5). There is also a suggestion that the effect produced on white rabbits (Group I) by living in an environment of neon light or in the dark is less than that shown by dark colored animals (Groups II and III).

The most interesting feature of the results obtained for the hypophysis is the difference between Group I on the one hand, and Groups II and III on the other (Table I and Text-fig. 6). The light and dark divisions of Groups II and III show the same relation to each other, while in Group I this relation is reversed. Group I also shows the

smallest values for control and light animals, while in Groups II and III animals of these classes give the largest values.

The suprarenals show a similar relation, so far as control animals are concerned, with a distinction between light and dark divisions that is clearly defined in all cases (Table I and Text-fig. 6). There is the suggestion here also that the smaller values for Group III as compared with Group II may have been due to some condition affecting control as well as experimental animals and, in the case of the dark division, to the persistence of an effect from the previous exposure to neon light.

The results for the testicles and pineal gland are irregular and of uncertain significance (Table I, Text-fig. 7). On the whole, it seems, however, that there is evidence of a tendency toward smaller values for animals in the light as compared with either the controls or the dark divisions of the same group and for the dark to be larger than the controls. It is the variation of results for control animals that is confusing.

The axillary and popliteal lymph nodes may be considered together as they are parts of the same system of organs (Table I, Text-fig. 8). The results obtained for the two groups of nodes agree in most respects. The animals in the light gave the largest values in all cases; the weights were either close to normal or above normal, while those of control and dark animals were subnormal with a relation between the two that was inconstant.

The deep lymph nodes, including the central mesenteric and deep cervical groups, show an entirely different situation (Table I and Text-fig. 9). The mesenteric nodes were extremely small in all cases, and in 8 of the 9 divisions the relations found agreed closely with those shown by the gastrointestinal mass. This agreement is to be expected as the weights of the two organs usually vary in the same direction.

The values for the deep cervical lymph nodes are of doubtful significance as several animals had infections of the nasal sinuses. The results are of interest, however, as an illustration of the disturbing influence of an extraneous factor.

The results have not been subjected to a detailed statistical analysis as the number of animals in each division (5) is too small to warrant such treatment. Reference to Table II will show, however, that in

many cases the differences found are sufficiently large to be regarded as significant when taken in conjunction with the trend of the apparent effect produced by a given type of environment.

Thus far, we have considered the results obtained for the weight of a given organ independent of the weight of any other organ, but it is evident that even large variations in the weights of organs may occur without materially changing the relations between organs, or that the relation of the weight of one organ to that of another may be greatly altered by slight changes in the weights of the organs concerned. This aspect of the problem of organic constitution is of even greater importance than the actual weights of organs or the weight of organs per unit of body weight, but we cannot undertake a detailed discussion of the results from this point of view as there are so many comparisons that might be made. A few examples will serve to show that the relations between organs were affected.

From the point of view of nutrition and growth the relation between the liver and the gastrointestinal mass is of interest. Normally, the weight of the liver per kilo of body weight is 1/5 or 20.00 per cent of that of the gastrointestinal mass (mean standard value, 20.41 per cent) and this relation is affected very little by differences in body weight *per se*. In these experiments, the ratio between the weight of the liver per kilo of body weight and that of the gastrointestinal mass, expressed in per cent, was as follows:

	Group I	Group II	Group III	Mean
Control	20.70	18.81	16.72	18.74
Light	18.06	18.30	17.60	17.98
Dark	16.27	16.98	18.30	17.18

The important points to be noted are the magnitude and the constancy of the values obtained. Thus the results for control animals were somewhat irregular, while the values for the light animals of Groups I and II were more constant and slightly smaller and those for the dark animals were still smaller. The values for Group III were apparently affected by the final change in environment as the figure for the dark division of this group is in absolute agreement with the

light division of Group II, while the figure for the light division is nearer that obtained for animals in the dark.

From the standpoint of metabolism, the relation between the thyroid and suprarenals is also of interest, as has been shown by Marine and his associates (7). Unlike the liver and gastrointestinal mass, the ratio between thyroid and suprarenal weights in normal rabbits is inconstant. The mean standard value (relative weights) is 60.76 per cent, but this value is subject to wide variation, as are the weights of the thyroid and suprarenals. The range of variation found on the basis of a body weight grouping of the normal rabbits studied by us was 47.94 to 77.35 per cent or a deviation of approximately 25.00 per cent in either direction from the mean value. The values obtained in these experiments were as follows:

	Group I	Group II	Group III	Mean
Control	63.49	63.79	69.54	65.61
Light	87.93	136.25	73.94	99.37
Dark	64.41	60.83	61.92	62.39

It is evident that these figures indicate a distinct difference in the thyroid-suprarenal equilibrium of the 3 classes of animals. The figure for the light division of the third group is again suggestive of the persistence of an effect following the change of environmental conditions, and there is also a suggestion of a difference between the conditions presented by white and by black animals of the light and dark divisions.

The values for the ratio of the hypophysis to the thyroid and for the thyroid to the thymus, and for the parathyroids to the thyroid are tabulated below as further examples of the effect of environmental conditions on organic equilibria.

Ratio of the Hypophysis to the Thyroid in Per Cent.

	Group I	Group II	Group III	Mean
Control	8.57	7.23	6.61	7.47
Light	6.48	3.92	9.27	6.56
Dark	8.34	8.71	8.55	8.53

Ratio of the Thyroid to the Thymus in Per Cent.

	Group I	Group II	Group III	Mean
Control	11.33	15.20	13.69	13.41
Light	15.64	27.35	13.07	18.70
Dark	11.56	13.00	10.85	11.80

Ratio of the Parathyroids to the Thyroid in Per Cent.

	Group I	Group II	Group III	Mean
Control	4.39	3.38	3.12	3.63
Light	3.80	1.65	4.25	3.23
Dark	4.13	4.41	5.91	4.82

Similar differences are shown by other organs. It is thus evident that the conditions under which the animals lived affected not only the weights of organs but the organic equilibrium in the broadest sense. Moreover, while the number of animals is too small to warrant a definite conclusion, it appears that there are some constitutional differences between black and white rabbits and that the two classes of animals do not show the same response to changes in environmental conditions. The results for the light and dark divisions of Group III do not agree in all respects with those for the corresponding divisions of Groups I and II; in some instances the results are reversed which suggests that the condition found at autopsy represents a transitional state referable to a change in environmental conditions.

In the present state of knowledge, it is difficult to correlate the size or weight of a given organ with a particular state of functional activity. This difficulty is increased by the fact that under certain circumstances, the relation may be direct, while under others it is inverse. Still, with due allowance for any uncertainty that may exist, there is sufficient evidence to warrant the conclusion that in these experiments there is a relation between the results obtained for organ weights on the one hand, and for increase in body weight and the growth of hair on the other. This is best shown by the results for the liver and for the ratio of the thyroid to the suprarenals. The results obtained for the thyroid-suprarenal relation agree with theoretical

expectations based on the work of Marine and his associates (7) and the results for the liver follow as a logical consequence of the activity of these two organs.

SUMMARY.

The influence of light environment on the organic constitution of normal rabbits was studied by comparing the weights of organs of animals that had been living under cerrtain conditions for long periods of time.

It was found that the light environment produced an effect on the physical constitution of the rabbits which was comparable to the effects produced on the functional activity of the same animals.

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CHEMICAL FINDINGS IN THE BLOOD OF THE DOG AFTER TEMPORARY OBSTRUCTION OF THE PYLORUS.

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MacCallum and his coworkers (1), and Hastings, Murray, and Murray (2) in studying pyloric obstruction in the dog noted a marked fall in chlorides and a rise in the CO_2 combining power of the blood. Similar observations in clinical cases were made by Grant (3). The close similarity of such changes with those found in high intestinal obstruction (4) led us to study the non-protein nitrogen of the blood in dogs with the pylorus obstructed (5). The non-protein nitrogen in such animals was found to reach the high levels characteristic of high intestinal obstruction. These observations have since been verified by others in both clinical (6) and experimental work (7, 8).

Our special interest in the subject was the relation of the chloride metabolism to the toxemia as reflected by the non-protein nitrogen level. The observation was made that the toxemia and rise in non-protein nitrogen in high intestinal obstruction do not usually occur until the chloride store of the blood is reduced below a certain level (4). It was also shown that the administration of sodium chloride had a remarkably beneficial effect on the toxemia of intestinal and pyloric obstruction and likewise in preventing the onset of a toxemia if given early (9). No beneficial effects were observed from water alone, other inorganic salts, or glucose. The therapeutic value of NaCl is now well accepted in both pyloric and intestinal obstruction. None of the explanations given for its favorable action is entirely satisfactory.

With the vomiting incident to pyloric obstruction much water and chloride are lost. This dehydration causes a diminution in kidney function with consequent retention of the end-products of protein metabolism. In the toxemias due to upper gastrointestinal tract

obstruction, there is a markedly accelerated nitrogen metabolism with increased tissue breakdown (10, 11). The rise in the non-protein nitrogen in the blood is often too rapid to be accounted for by simple retention. The high level of non-protein nitrogen seems due to both retention and increased tissue destruction.

The exact relation of the chlorides to the toxemia and tissue destruction is as yet undetermined. We suggested originally the possibility that sodium chloride may directly neutralize the bodies responsible for the toxemia. This hypothesis was based on the seeming greater loss of chloride from the blood than could be accounted for in the vomitus and urine and on the striking effect of sodium chloride in relieving and preventing the toxemia. Further studies by Gamble and McIver (8), White and Bridge (12), and Atchley and Benedict (13) indicate that all the chloride lost from the blood and tissues is found in the urine and gastric and intestinal secretions. There is also a fall in the chloride of the tissues (12, 14), and during the toxemia very little chloride is excreted in the urine. These facts render our original theory unlikely.

This still however does not throw any light on the problem of the relation of the chloride level to the tissue metabolism. The proper amount of chloride is necessary to maintain water balance and kidney function. Autolysis may conceivably be more rapid in tissue with a low chloride content. It is also possible that the tissues may be more easily injured by circulating toxins, if such be present, when the salt content is low. These experiments have been made in an attempt to throw some light on the exact relation of the chloride level to the toxemia of upper gastrointestinal tract obstruction.

In studying the relation of the sodium chloride to the blood chemical changes and toxemia, it is most desirable to be able to produce experimentally the characteristic blood changes and toxemia without having to consider mechanical factors, the possibility of infection, and operative risk. We have found that such a condition can be produced by ligating the pylorus and releasing the obstruction in 24 to 72 hours. The thick wall of the pylorus precludes the possibility of rupture of the viscus as frequently happens after ligation of the intestine. The obstruction is easily eliminated by removing the ligature under local anesthesia with little operative risk. In some of the surviving animals

there is produced a characteristic toxemia in which mechanical factors are eliminated making an ideal condition for the study of the blood chemical changes.

Method.

All experiments have been made on dogs. All primary operations were done under ether anesthesia with aseptic technique. Obstruction of the pylorus was

TABLE I.

Typical Changes in Blood of Dog after Simple Obstruction of Pylorus.

Dog No.	Blood					Remarks
	Amount per 100 cc.				CO_2 combining power	
	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	*mg.*	*mg.*	*vol. %*	
1	0	35.7	11.2	460	24.0	Pylorus obstructed
	1	46.0	14.5	320	52.0	
	2	111.0	49.0	230	37.7	Died
2	0	37.5	7.9	500	35.4	Pylorus obstructed
	1	38.9	15.4	470	30.5	
	2	57.3	30.8	360	50.2	
	3	129.0	70.0	310	56.0	Died
3	0	28.9	12.6	490	27.5	Pylorus obstructed
	1	27.0	11.2	450	44.7	
	2	61.0	25.2	380	42.8	
	3	76.5	28.7	340	53.9	
	4	181.0	128.2	260	45.3	Died
4	0	33.7	12.6	500	30.5	Pylorus obstructed
	1	46.0	21.0	480	36.2	
	2	155.0	47.6	330	29.4	Died

effected by ligating with a piece of narrow tape. The tape was fixed to the anterior abdominal wall so that it could be easily located at the second operation. The obstruction was released by making a short incision under local anesthesia, cutting and removing the tape. At autopsy after such a procedure no permanent anatomic changes in the pylorus could be observed. The most desirable time for the second operation is usually 48 hours after the obstruction is made. The dogs were kept in metabolism cages. No food was given during the experiment and usually none for 48 hours before operation. Water was allowed *ad libitum*.

TABLE II.

Course of Toxemia in Dogs Recovering after Temporary Obstruction of Pylorus without Treatment.

Dog No.	Blood					Remarks
	Amount per 100 cc.				CO_2 combining power	
	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	*mg.*	*mg.*	*vol. %*	
5	0	37.5	10.3	480	30.5	Pylorus obstructed
	1	34.5	8.4	440	40.9	
	2	61.0	26.6	350		Obstruction released
	3	79.0	30.1	310	54.9	
	4	50.0	9.8	330	36.2	
	5	27.3	18.9	330	40.0	
	6	26.3	15.4	370	56.0	
	7	30.0	9.1	360	40.9	
	8	31.2	10.7	380	49.4	
	9	30.3	6.5	430	42.8	
	10	27.3	7.5	460	48.5	Well
6	0	27.7	13.1	460	41.9	Pylorus obstructed
	1	37.5	14.7	370	50.4	
	2	65.5	30.8	330	58.7	
	3	42.8	14.0	330	56.8	
	4	40.0	16.8	340	59.6	
	5	39.5	16.8	340	56.5	
	6	40.0	18.2	340	47.5	
	7	37.5	17.5	350	55.8	
	9	38.0	9.1	370	57.7	
	10	30.9	8.4	400	52.2	
	11	33.0	10.7	420	48.5	
	12	57.3	7.5	450	51.3	Well
7	0	28.5	13.1	500	38.1	Pylorus obstructed
	1	25.9	9.8	440		
	2	30.0	9.1	440	41.9	
	3	33.0	16.1	380	40.0	Obstruction released
	4	50.8	22.9	330	53.2	
	5	34.5	20.7	370	61.7	
	6	42.8	23.7	370	57.9	
	7	125.0	73.6	330	57.7	
	8	32.3	9.1	380	53.9	
	9	31.6	16.1	410	50.2	
	10	42.2	14.0	390	67.1	
	11	32.3	11.7	420	57.7	
	12	41.0	15.4	400	46.6	
	13	50.0	13.1	420	58.7	Well

TABLE II—*Concluded.*

Dog No.	Blood					Remarks
	Amount per 100 cc.					
	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. %*	
8	0	24.6	7.7	450	37.2	Pylorus obstructed
	1	28.0	16.8	400	40.0	
	2	89.0	52.5	330	41.0	Obstruction released
	3	72.3	40.6	320	38.1	
	4	34.5	17.5	380	36.2	
	6	40.0	18.2	420	35.3	
	7	38.0	16.1	400	31.5	
	8	34.5	14.7	420	43.8	
	9	30.0	9.1	440	39.0	
	10	29.4	17.5	440	41.9	Well

Blood was obtained for analysis from the jugular vein just before operation and at 24-hour intervals thereafter.

The non-protein nitrogen of the blood was determined by the method of Folin and Wu (15), urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (16), CO_2 combining power by the method of Van Slyke (17), and the chlorides in the tungstic acid filtrate after the technique suggested by Gettler (18). For the non-protein nitrogen in the urine Folin's micro method (19) was used, and for the chlorides a modified Volhard-Arnold method.

EXPERIMENTAL OBSERVATIONS.

The blood chemical changes in four dogs in which the obstruction was not released are shown in Table I. These all show the characteristic findings, namely, a fall in chloride and a rise in CO_2 combining power, and in non-protein and urea nitrogen. The animals died in 48 to 96 hours after operation.

In eight dogs the obstruction was released but no treatment was given. Four recovered, and four died. In the animals recovering (Table II) there was a gradual rise in the blood chloride until the normal level was reached. No food was given during the experiment so it is quite evident that there was a shift in the store of body chloride so that the diminished supply of blood chloride was restored. The four dogs which died showed a progressive fall in chloride and rise in

TABLE III.

Effect of Sodium Chloride Given by Mouth on Toxemia Induced by Temporary Obstruction of Pylorus.

Dog No.	Blood					Remarks
	Amount per 100 cc.				CO_2 combining power	
	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	*mg.*	*mg.*	*vol.* %	
9	0	34.9	15.4	430	32.4	Pylorus obstructed
	2	31.6	14.0	330	46.6	
	3	30.0	16.1	300	60.0	Obstruction released
	4	33.3	17.5	310	50.2	
	5	37.5	20.3	290	55.0	
	6	42.2	12.6	320	53.6	
	7	60.0	18.2	360	59.6	
	9	79.0	35.0	360	40.0	
	10	67.5	37.8	320	52.0	10 gm. NaCl by mouth
	11	38.9	21.0	350	45.7	10 gm. NaCl by mouth
	12	23.3	10.5	470	41.9	10 gm. NaCl by mouth
	13	25.7	15.4	500	41.9	Well
10	0	30.9	14.0	470	28.7	Pylorus obstructed
	1	31.6	13.8	450	38.1	
	2	39.5	14.0	360	39.0	
	3	111.0	52.5	300	53.9	Obstruction released
	4	67.0	39.3	350	45.7	
	5	60.0	36.4	310	46.6	
	6	55.0	30.8	340	47.5	
	7	51.8	31.5	350	53.0	
	8	70.0	38.5	340	52.2	
	9	72.8	48.3	340	52.0	
	10	79.0	49.0	370	50.2	10 gm. NaCl by mouth
	11	34.1	21.7	390	57.7	10 gm. NaCl by mouth
	12	34.9	20.3	470	39.0	
	13	34.1	19.6	470	52.0	
	14	33.0	19.6	500	48.5	
	15	39.5	23.8	470	50.2	
	16	28.5	16.1	520	46.6	
	17	27.0	17.5	600	40.0	Well
11	0	61.0	26.6	510	30.5	Plyorus obstructed
	1	68.8	35.0	500	38.1	
	2	117.0	51.1	400	59.6	Obstruction released
	3	137.0	75.7	370	55.8	
	4	123.0	56.7	350	49.4	
	5	120.0	60.2	380	45.7	
	6	120.0	64.4	400	51.3	
	7	100.0	55.3	400	53.9	

TABLE III—*Concluded.*

Dog No.	Blood					Remarks
	Amount per 100 cc.				CO_2 combining power	
	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	*mg.*	*mg.*	*vol. %*	
12	8	103.0	59.5	380	51.3	
	9	115.0	57.4	390	47.4	
	10	98.8	45.5	400	55.8	
	11	143.0	82.9	380	28.7	
	12	100.0	50.4	420	45.7	
	13	126.0	60.2	400	36.2	10 gm. NaCl by mouth
	14	88.4	44.1	480	4.0	10 gm. NaCl by mouth
	15	60.0	23.1	440	30.5	
	16	71.3	43.9	530	32.4	
	17	57.3	35.0	570	31.5	
	18	42.2	15.4	580	24.0	
	19	35.7	14.0	520	28.7	
	20	38.9	19.6	480	30.5	Well
13	0	51.8	26.2	430	42.1	Pylorus obstructed
	1	30.3	12.6	340	67.1	
	2	68.2	43.3	210	72.7	Obstruction released
	3	132.2	72.9	260	74.5	
	4	83.3	57.1	230	80.1	
	5	77.3	38.2	270	74.5	
	6	107.0	63.9	260	74.5	
	7	158.0	98.8	350	56.9	
	8	208.0	123.3	290	58.8	
	9	173.0	100.8	250	67.5	
	10	230.0	141.5	270	65.3	
	11	250.0	159.7	270	71.8	10 gm. NaCl by mouth
	12	136.0	78.5	410	61.6	Died immediately after intravenous injection of 25 per cent NaCl

non-protein nitrogen after the obstruction was released. The course of the toxemia was not influenced by the release of the obstruction.

Five animals were treated with sodium chloride by mouth after a toxemia had been established for a sufficiently long period to indicate that it would not adjust itself spontaneously (Tables III and IV). The sodium chloride was given by mouth in 1 gm. tablets with a small

TABLE IV.

Blood and Urine Findings after Temporary Obstruction of the Pylorus.

Dog No.	Day after operation	Blood					Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Hematocrit (% plasma)	Amt.	Chlorides		Nitrogen		
		mg.	mg.	mg.	vol. %	%	cc.	%	gm.	%	gm.	
14	0	44.0	10.3	470	38.1	51						Operation
	1	28.0	9.3	430	45.7	41						
	2	125.0	36.0	470	49.4	42	250	0.50	1.50	0.96	2.40	Obstruction released
	3	175.0	92.2	450	50.2	43	900	0.01	0.09	0.33	2.97	
	4	276.0	126.6	380	40.0		320	0.01	0.032	1.38	4.4	
	5	220.0	132.6	370	40.9	53	680	0.01	0.068	0.44	2.99	
	6 a.m.	236.0	154.1	370	41.9	58	560	0.22	1.23	0.68	3.80	100 cc. 10 per cent NaCl by stomach tube
	6 p.m.	169.0	98.5	430	38.1	59						
	7	78.0	57.0	490	38.1	61	1330	0.63	8.38	0.68	9.0	
	8	75.0	47.2	450	32.4	67	270	0.11	0.30	2.00	5.4	
	9	27.7	17.3	490	38.1	65	240	0.06	0.14	2.50	6.0	
	10	24.4	14.9	480	38.1	72	140	0.07	0.09	3.30	4.6	
	11						190	0.15	0.29	2.85	5.4	
	12	28.1	8.9	580	34.4		150	0.35	0.53	2.00	3.0	Recovered
	13											
15	0	33.0	8.4	470	34.3	55						Operation
	2	68.3	33.6	420	48.3	42						
	3	132.0	98.1	430	50.2	52	730	0.35	2.56	0.64	4.67	Obstruction released
	4	173.0	112.8	320	47.5	48	340	0.07	0.24	1.77	6.02	
	5	159.0	91.1	440	41.6		275	0.05	0.14	3.00	8.25	
	6						355			3.00	10.65	
	7		44.8	410	45.7		195	0.03	0.06	3.00	5.05	
	8	37.5	23.8	410	52.0		220	0.03	0.06	1.02	4.22	
	9	40.6	16.8	410	55.8		220					
	10	29.1	14.0	450	59.6		210	0.05	0.11	1.00	2.1	Recovered
16	0	30.0	13.3	490	38.1							Operation
	1	30.3	12.4	420	41.9							
	2	59.5	38.5	330	57.7		1000	0.05	0.50	0.92	9.2	Obstruction released
	3	41.6	15.4	390	52.0		625	0.05	0.31	1.23	7.7	
	4	41.0	22.4	380	48.8		610	0.05	0.30	1.04	6.3	
	5	32.6	16.1	380	52.0		500	0.02	0.10	1.92	9.6	
	6	38.9	14.0	390	50.2		235	0.03	0.70	3.00	7.05	
	7	31.9	15.4	400	55.8		365	0.06	0.22	1.44	5.3	Recovered
	8	27.0	16.8	400	54.6		265	0.03	0.08	1.10	2.9	

amount of water in four animals and in 10 per cent solution in another. A very large amount of salt was required for each animal. The four recovering showed a rapid return of blood findings to normal, and a disappearance of the signs of toxemia. The animal which did not recover showed a marked fall in non-protein nitrogen after the administration of 10 gm. of salt by mouth. The following morning an intravenous injection of 25 per cent sodium chloride was attempted with immediate death of the animal.

In three animals chemical studies were also made on the urine after the pyloric obstruction was released (Table IV). After the release there was no vomiting, so accurate urine collections could be made. Distilled water was allowed *ad libitum*. No food was given. In one animal (No. 13) the non-protein nitrogen continued to rise and the chlorides to fall after the removal of the obstruction. There was a marked diuresis, with a high nitrogen and low chloride excretion. Even with this marked diuresis and high nitrogen output the non-protein nitrogen in the blood continued to rise indicating the continuation of the increased protein destruction. The hematocrit reading before operation was 51, and 4 days after the release operation it was 58. When the non-protein nitrogen was 236 mg. per 100 cc. the animal was given 100 cc. 10 per cent sodium chloride by stomach tube in broken doses over several hours time. In the afternoon the non-protein nitrogen had fallen to 169 mg. with practically no change in the hematocrit reading. The following day there was a marked diuresis with a high nitrogen excretion. Almost all the chloride given was excreted within 48 hours. The non-protein nitrogen rapidly returned to normal and remained so. In two other animals there was also a marked diuresis after release of the obstruction. The nitrogen excretion was very high. The non-protein nitrogen returned to normal without treatment. The blood chloride returned to normal in one animal and almost to normal in the other.

DISCUSSION.

Temporary obstruction of the pylorus is a procedure by which the characteristic toxemia of upper gastrointestinal tract obstruction may be produced without having to consider mechanical factors, infection, or operative risk. It gives an opportunity for studying the toxemia factor alone.

The animals which recovered after the release of the obstruction showed the findings now known as characteristic of pyloric obstruction. The animals which recovered spontaneously all showed a quite low blood chloride at the height of the toxemia. The lowest level of chloride always occurred with the highest non-protein nitrogen. As the non-protein nitrogen returned to normal the chloride rose and gradually returned to normal with the animal receiving nothing by mouth except distilled water. These results can be explained only on the basis of a redistribution of the body chlorides. Some of the chloride lost from the blood must have been stored somewhere within the body only to return to the blood after the toxemia is relieved. The level of chloride in the blood is evidently not a true index of the chloride in the body.

The effect of sodium chloride given by mouth in the toxic animals was immediate and most striking. In no instance was the salt given until it was quite apparent that the toxemia would not be relieved by water alone. In every animal so treated there was a rapid return of the blood to normal except the one in which a fatal result followed a later intravenous injection.

The study of the urine after temporary obstruction of the pylorus emphasizes again the high nitrogen excretion which can be explained only on the basis of increased protein destruction. There is a marked diuresis after the obstruction is released probably due to the increased urea content of the blood. The administration of sodium chloride caused a transient increase in the diuresis and output of nitrogen and salt with a rapid return to normal. The assumption seems justified that the restoration of the chlorides has stopped the increased protein destruction.

CONCLUSIONS.

The toxemia characteristic of upper gastrointestinal tract obstruction may be produced by temporary obstruction of the pylorus. This procedure affords an opportunity for studying the toxemia in the absence of mechanical factors, operative risk, and infection.

Animals which spontaneously recover from the toxemia may show a return of the blood chloride to normal when only distilled water is given. In such instances there must be a redistribution of the body store of chlorides.

The administration of sodium chloride by mouth to animals which show a toxemia without evidence of spontaneous recovery causes a rapid return of the blood to normal.

There is a marked diuresis with high nitrogen excretion during the toxemia. This is evidently due to the increased protein destruction.

The return of the blood chlorides to normal causes a cessation of the increased protein destruction.

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